Associations between environmental or behavioural factors and natural history of MS

By

Chunrong Tao

Submitted in fulfilment of the requirements for the Degree of
Doctor of Philosophy (Medical Research)

UNIVERSITY OF TASMANIA

Menzies Institute for Medical Research Tasmania

University of Tasmania

October, 2018

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by any other person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Signed: Date: 30/10/2018

Statement of Ethical Conduct

"The research associated with this thesis abides by the international and Australian codes on human experimentation, and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University."

The Human Research ethics Committee (Tasmania) Network approved this project (Approval number H11613: Strategies to address the long term maintenance of bone density in younger women: fracture risk feedback and vitamin D). We obtained written informed consent from all participants.

Signed: Date: 30/10/2018

Statement of authority of access and regarding published work

This thesis can be made available for loan. Copying of any part of this thesis is prohibited for two years from the date this statement was signed; after that time limited copying is permitted in accordance with the Copyright Act 1968.

The publishers of the papers comprising Chapters 3, 4 and 5 hold the copyright for that content, and access to the material should be sought from the respective journals.

Signed: Date: 30/10/2018

Statement of Co-authorship

Author details and their roles:

Paper 1, Association between human herpesvirus & human endogenous retrovirus and MS onset & progression:

Chunrong Tao, Steve Simpson, Jr., Bruce V Taylor, Ingrid van der Mei
Menzies Institute for Medical Research, University of Tasmania

Located in Chapter 3

Candidate was the primary author and undertook the literature review and composed the initial draft with direct assistance from Ingrid van der Mei (IvM). Candidate contributed approximately 75% to the planning, execution and preparation of the work for the paper.
Bruce Vivian Taylor (BVT) and Steve Simpson Jr. (SSJ) provided supervision and were involved in the critical revision of the manuscripts.

Paper 2, Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis:

Chunrong Tao[1], Steve Simpson, Jr.[1], Ingrid van der Mei[1], Leigh Blizzard[1], Eva Havrdova[2], Dana Horakova[3], Vahid Shaygannejad[4], Alessandra Lugaresi[5], Guillermo Izquierdo[6], Maria Trojano[7], Pierre Duquette[8], Marc Girard[8], Franois Grand'Maison[9], Pierre Grammond[10], Raed Alroughani[11], Murat Terzi[12], Celia Oreja-Guevara[13], Seyed Aidin Sajedi[14], Gerardo Iuliano[15], Patrizia Sola[16], Jeannette Lechner-Scott[17], Vincent Van Pesch[18], Eugenio Pucci[19], Roberto Bergamaschi[20], Michael Barnett[21], Cristina Ramo[22], Bhim Singhal[23], Daniele LA Spitaleri[24], Mark Slee[25], Freek Verheul[26], Ricardo Fernández Bolaños[27], Maria Pia Amato[28], Edgardo Cristiano[29], Franco Granella[30], Suzanne Hodgkinson[31], Marcela Fiol[32], Orla Gray[33], Pamela McCombe[34], Maria Laura Saladino[35], José Luis Sánchez Menoyo[36], Neil Shuey[37], Steve Vucic[38], Cameron Shaw[39], Norma Deri[40], Walter Oleschko Arruda[41], Helmut Butzkueven[42, 43], Tim Spelman[42], Bruce V Taylor[1], on behalf of the MSBase Study Group.

[1] Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia, [2] Department of Neurology and Center of Clinical Neuroscience, 1st Faculty of Medicine, General University Hospital and Charles University in Prague, Czech Republic, [3] Department of Neurology and Center of Clinical Neuroscience, Charles University in Prague, First Faculty of Medicine and General University Hospital, Prague, Czech Republic, [4] Al-Zahra Hospital, Isfahan University of Medical Sciences,

Isfahan, Iran, [5] MS Center, Department of Neuroscience, Imaging and Clinical Sciences, University "G. d'Annunzio", Chieti, Italy, [6] Hospital Universitario Virgen Macarena, Sevilla, Spain, [7] Department of Basic Medical Sciences, Neuroscience and Sense Organs, University of Bari, Bari, Italy, [8] CHUM – Hôpital Notre Dame, Montreal, Canada, [9] Neuro Rive-Sud, Hôpital Charles LeMoyne, Quebec, Canada [10] CISSS Chaudiere-Appalaches, Levis, Quebec, Canada, [11] Amiri Hospital, Kuwait City, Kuwait, [12] Department of Neurology, Mayis University, Samsun, Turkey [13] University Hospital San Carlos, IdISSC, Madrid, Spain, [14] Golestan Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [15] Ospedali Riuniti di Salerno, Salerno, Italy, [16] Department of Neuroscience, Universitu of Modena e Reggio Emilia, Nuovo Ospedale Civile S. Agostino/Estense, Modena, Italy.[17] John Hunter Hospital, University of Newcastle, Hunter Medical Research Institute, Newcastle, Australia, [18] Cliniques Universitaires Saint-Luc, Brussels, Belgium, [19] Neurology Unit, ASUR Marche – AV 3, Macerata, Italy, [20] C. Mondino National Neurological Institute, Pavia, Italy, [21] Brain and Mind Research Institute, Sydney, Australia, [22] Hospital Germans Trias I Pujol, Badalona, Spain, [23] Bombay Hospital Institute of Medical Sciences, Mumbai, India, [24] AORN San Giuseppe Moscati Avellino, Avellino, Italy, [25] Flinders University and Medical Centre, Adelaide, Australia, [26] Groene Hart Ziekenhuis, Gouda, Netherlands, [27] Hospital Universitario Virgen de Valme, Sevilla, Spain, [28] Department of NEUROFARBA, Section of Neurosciences, University of Florence, Florence, Italy, [29] Hospital Italiano, Buenos Aries, Argentina, [30] Department of Neuroscience, University of Parma, Parma, Italy, [31] Liverpool Hospital, Liverpool, Australia, [32] FLENI, Buenos Aries, Argentina, [33] Ulster Hospital, Dundonald, U.K. [34] St Andrew's Place, Brisbane, Australia, [35] INEBA, Buenos Aries, Argentina, [36] Hospital de Galdakao-Usansolo, Galdakao, Spain, [37] St Vincent's Hospital, Melbourne, Australia, [38] Westmead Hospital, Sydney, Australia, [39] Geelong Hospital, Geelong, Australia, [40] Hospital Fernandez, Capital Federal, Argentina, [41] Hospital Ecoville, Curitiba, Brazil, [42] Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Melbourne, Australia, [43] Departmnet of Neurology, Eastern Health, Monash University, Box Hill, Australia

Located in Chapter 4

Candidate was the primary author and analysed the data, wrote the draft manuscript and completed revisions. Candidate contributed approximately 70% to the planning, execution and preparation of the work for the paper.
IvM and SSJ assisted with data analysis and provided statistical advice.
BVT designed and conceptualised the study.
EH, DH, VS, AL, GI, MT,PD, MG, FG'M, PG, RA, MT, CO-G, SAS, GI, PS, JL-S, VVP, EP, RB, MB, CR, BS,DLAS, MS, FV, RFB, MPA, EC, FG, SH, MF, OG, PMC, MLS, JLSM, NS, SV, CS, ND,WOA and HB all participated in data collection.
All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and read and approved the final manuscript.

Paper 3, Tobacco smoking, progressive-onset, cerebral dysfunction are associated with a delayed FDE onset and marijuana use with an earlier onset:

Chunrong Tao [1], Steve Simpson, Jr. [1,2],Bruce Taylor[1], Leigh Blizzard[1], Robyn M Lucas[3], Anne-Louise Ponsonby[4], Simon Broadley[5], AusLong/Ausimmune Investigators group[6], Ingrid van der Mei[1]

[1]Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia; [2] Institute for Health & Ageing, Australian Catholic University, Melbourne, Australia; [3]National Centre for Epidemiology and Population Health, Canberra, Australia; [4]Murdoch Children's Research Institute, University of Melbourne, Melbourne, Australia; [5]School of Medicine, Gold Coast Campus, Griffith University QLD, Australia; [6]A full list of members is provided in the Acknowledgments.

Located in Chapter 5

Candidate was the primary author and analysed the data, wrote the draft manuscript and completed revisions. Candidate contributed approximately 70% to the planning, execution and preparation of the work for the paper.
SSJ and LB assisted with data analysis and provided statistical advice, and were involved in the critical revision of the manuscript.
IvM and BVT designed this study and formulated the hypotheses for this analysis.
All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and read and approved the final manuscript.

Paper 4, Associations between immune responses to Epstein-Barr virus and Human Herpes Virus 6 and multiple sclerosis clinical course:

Chunrong Tao [1], Steve Simpson, Jr. [1],Bruce Taylor[1], Leigh Blizzard[1], Robyn Lucas[2], Anne-Louise Ponsonby[3], Simon Broadley[4], AusLong/Ausimmune Investigators group[5], Ingrid van der Mei[1]

[1]Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia; [2]National Centre for Epidemiology and Population Health, Research School of Population Health, Australian National University, Canberra, Australia; [3]Murdoch Children's Research Institute, University of Melbourne, Melbourne, Australia; [4]School of Medicine, Gold Coast Campus, Griffith University QLD, Australia; [5]A full list of members is provided in the Acknowledgments.

Located in Chapter 6

Candidate was the primary author and analysed the data, wrote the draft manuscript and completed revisions. Candidate contributed approximately 70% to the planning, execution and preparation of the work for the paper.

SSJ and LB assisted with data analysis and provided statistical advice, and were involved in the critical revision of the manuscript.
IvM and BVT designed this study and formulated the hypotheses for this analysis. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and read and approved the final manuscript.

Paper 5, Positive stressful life events are associated with a lower hazard of relapses in early multiple sclerosis:

Chunrong Tao[1], Steve Simpson, Jr.[1],Bruce Taylor[1], Leigh Blizzard[1], Robyn M Lucas[2,3], Anne-Louise Ponsonby[4], Jenn Scott[5], Brenda Wood[1], AusLong/Ausimmune Investigators group[6], Ingrid van der Mei[1]

[1]Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia; [2]National Centre for Epidemiology and Population Health, Research School of Population Health, Australian National University, Canberra, Australia; [3] Centre for Opthalmology and Visual Science, University of Western Australia, Perth, Australia [4]Murdoch Children's Research Institute, University of Melbourne, Melbourne, Australia;[5] School of Medicine (Psychology) , University of Tasmania , Hobart , Australia. [6]A full list of members is provided in the Acknowledgments.

Located in Chapter 7

Candidate was the primary author and analysed the data, wrote the draft manuscript and completed revisions. Candidate contributed approximately 70% to the planning, execution and preparation of the work for the paper.
SSJ and LB assisted with data analysis and provided statistical advice, and were involved in the critical revision of the manuscript.
IvM and BVT designed this study and formulated the hypotheses for this analysis. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and read and approved the final manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed:

	Ingrid van der Mei	James Sharman, Deputy Director for Alison Venn
	Supervisor	Head of School
	Menzies Institute for Medical Research	Menzies Institute for Medical Research
	University of Tasmania	University of Tasmania
Date:	31/10/2018	31/10/2018

Abstract

The aetiology of multiple sclerosis (MS) is unclear, but an aberrant immune response to Epstein-Barr virus (EBV) and infectious mononucleosis,a history of tobacco smoking, insufficient exposure to ultraviolet radiation, or decreased levels of 25-hydroxy vitamin D [25(OH)D] contribute to the onset in genetically susceptible individuals. Other factors such as human herpesvirus 6 (HHV6) infection, stressful life events (SLEs), number of offspring, and marijuana use may be associated with MS onset, but the evidence is currently less consistent. The effects of these factors on the age of symptom onset (ASO) and clinical course of patients with MS are also still uncertain.

This thesis used three databases to investigate the roles of these established/potential risk factors in ASO and the clinical course of MS. MSBase (chapter 4) is an international neurologist database, which I used to assess whether a latitudinal gradient of ASO exists in MS patients. The Ausimmune study (chapter 5) is a population-based incident case-control study conducted in four different locations in eastern Australia that I used to assess whether environmental/behavioural factors could influence the ASO in MS patients. The AusLong study (chapters 6 and 7) is a prospective cohort study that followed cases in the Ausimmune Study for at least 5 years; I used the latter to assess whether HHV6/EBV infection and SLEs could influence MS clinical course.

Within MSBase, including 22,162 patients from 21 countries, I found an inverse association between latitude and ASO, such that each 10 °of increased latitude was

associated with a 0.8-year earlier onset. Variation of ultraviolet radiation may play an important role in this latitudinal gradient. Using the Ausimmune Study, I showed that higher offspring number, a history of tobacco smoking, cerebral dysfunction at their first clinical diagnosis of CNS demyelination (FCD), and a primary progressive MS (PPMS) onset type were associated with a later onset, whereas a history of marijuana use was associated with an earlier onset. Both of these analyses supported the effects of modifiable factors on the age of symptom onset in MS, which may help improve surveillance and diagnostic efforts, as well as aid in planning for future case load.

Using the AusLong Study, I found little evidence supporting associations between immune response to EBV/HHV6 and MS clinical course, indicating that the viral infection repertoire detected at baseline could not predict the subsequent disease course or prognosis of MS. However, using this study I found a protective effect of perceived positive SLEs on MS disease activity, such that preceding perceived positive SLEs (number, severity, and duration) were associated with a decreased hazard of subsequent conversion to MS or relapses. These results support the important role of maintaining a positive psychological well-being in reduction of the frequency of disease activity.

The findings of the significant associations between environmental/behavioural factors and age of symptom onset and disease activity of MS, if replicated in other studies, will be useful for the prediction of MS onset and prognosis.

Personal acknowledgements

I thank my wonderful primary supervisor, Associate Professor Ingrid van der Mei, whom I respect immensely. I still remember the first time I met her when she told me I could just come to see her whenever I have any questions, and indeed I have been extremely lucky to have a supervisor who responded to my questions and doubts so promptly. In the past four years, she has brought me to the field of medical research and continued to encourage and enlighten me. She has given generously of her time, advice, wisdom, and this is of particular importance to an overseas student. I thank her for the substantial contributions she has provided throughout my time as her PhD student.

I also thank my co-supervisors, Professor Bruce Taylor and Dr. Steve Simpson, for their patience and time given to me in this my early medical research career. With their input, I have been able to have a much better understanding of many research methods, allowing me to form medical research questions from different perspectives. I am also very grateful for their guidance in strengthening my statistical skills so I can be a well-rounded researcher.

I thank my wife and daughterfor their continued support and encouragement, and for giving me their unconditional love. No matter where I am and what I am doing, they have always been standing by my side. They are everything to me.

Chunrong Tao

November 2017

Financial acknowledgements

I thank Tasmania Graduate Research Scholarships for giving me financial support during my candidature.

This study in chapter 4 was supported by MSBase foundation. The MSBase Foundationis a not-for-profit organisation that receives support from Merck Serono, BiogenIdec, Novartis Pharma, Bayer-Schering, Sanofi-Aventis and BioCSL.

The study in chapter 5, 6 & 7 were supported by was supported by the National Health and Medical Research Council of Australia (Grant reference number: 54922).

List of publications

Tao C, Simpson Jr S, Taylor BV, van der Mei I. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. Journal of the neurological sciences. 2017;372:239-249.

Tao C, Simpson S, Jr., van der Mei I, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. Journal of neurology, neurosurgery, and psychiatry. 2016;87(12):1343-1349.

Tao C, Simpson S, Jr., van der Mei I, et al. Onset Symptoms, Tobacco Smoking, and Progressive-Onset Phenotype Are Associated With a Delayed Onset of Multiple Sclerosis, and Marijuana Use With an Earlier Onset. Front Neurol. 2018;9:418.

Scientific presentations

Multiple Sclerosis Research Australia (MSRA) 2015 annual meeting, Melbourne (poster presentation);

European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) 2015 annual meeting, Barcelona, Spain (poster presentation);

International Conference on Neurology & Epidemiology2015 annual meeting, Gold Coast (oral presentation);

European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) 2016 annual meeting, London, England (poster presentation);

Multiple Sclerosis Research Australia (MSRA) 2017 annual meeting, Sydney (3 poster presentations and 1 oral presentation).

List of abbreviations

ASO	Age of symptom onset
BMI	Body mass index
CAMs	Complementary and alternative treatments
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DMT	Disease modifying therapy
DSS	Disability Status Scale
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
FDE	First demyelinating event
HHV6	Human herpesvirus 6
HR	Hazard ratio
IM	Infectious mononucleosis
IRR	Incidence rate ratio
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSSS	Multiple Sclerosis Severity Score
OR	Odds ratio
RCT	Randomised controlled trial
RR	Relative risk
RRMS	Relapsing-remitting MS
SD	Standard deviation
SLEs	Stressful life events
SNPs	Single nucleotide polymorphisms
SPMS	Secondary-progressive MS
UVR	Ultraviolet radiation

Table of contents

Chapter 1 Introduction

1.1 Multiple sclerosis and its impact

Multiple sclerosis (MS), a chronic autoimmune disease of the central nervous system (CNS), is the primary cause of neurological disability in young adults[1]. Improvements in diagnostic criteria and paraclinical evidence on magnetic resonance imaging (MRI) have improved the accuracy of MS frequency estimates generally, but particularly in non-Asian European-descent countries. The improved accuracy of frequency estimates is responsible for increasing trends in the prevalence and incidence of MS that have been found globally during the last few decades. According to the Atlas of MS database 2013[2], there are 2.3 million people with MS worldwide. With disease onset usually occurring during the third or fourth decade of life but with low case fatality rates (1%–4% per year), MS is associated with significant personal morbidity and economic burden[3 4]. Its impact on the economy in Europe alone is more than 15 billion euros, including direct medical costs and loss of productivity[4]. For patients with MS, quality of life is affected considerably[5-9]. MS has a highly variable inter- and intra-personal clinical course, both in pattern and rate of neurological deterioration, strongly suggesting multiple contributory factors that include both genetic and environmental influences.

1.2 Main objectives of this thesis

Studies about the aetiology of MS have advanced markedly during the last decade: environmental factors such as a higher immune response to Epstein-Barr virus (EBV) and a history of infectious mononucleosis (IM)[10], tobacco smoking[11], lower levels of

serum vitamin D[12], and insufficient exposure to ultraviolet radiation (UVR)[13] have been consistently associated with MS onset. Some other environmental/behavioural factors, such as parity, marijuana use, and stressful life events (SLEs), have shown a potential relationship with MS onset. However, the nature of these effects on age of symptom onset (ASO) and clinical course of MS is still uncertain.

My aims in this thesis are:

1. To examine whether a higher latitude of residence in patients with MS is associated with an earlier ASO, after controlling for factors such as sex, type of onset, and some distinct characteristics in each region such as mean age of the population, sex ratio, and MS onset type ratio (data source: MSBase).
2. To examine whether sex, latitude, UVR, serum vitamin D, tobacco smoking, marijuana use, offspring number, age at menarche, and clinical presentation features are associated with ASO (data source: Ausimmune Study).
3. To examine whether a higher immune response to EBV/human herpesvirus 6 (HHV6) infection at baseline is associated with an increased hazard of conversion to MS, relapses, and disability progression after adjustment for confounding factors (data source: AusLong Study).
4. To examine the association between SLEs and MS clinical course, and explore the different effects between perceived positive and negative SLEs (data source: AusLong Study).

1.3 The projects central to this thesis

To examine the association between latitude and ASO, an international neurologist database, MSBase, was used. This study[14] commenced in July 2004 and, by the time we started our analysis, 30,415 patients from 26 countries were recruited in the study.

The Ausimmune Study[15], funded by the US MS Society, the National Health and Medical Research Council (NHMRC), and MS Research Australia, was a population-

based, multicentre incident case-control study. It included the following regions: Brisbane city, the Newcastle region, Geelong and the Western Districts of Victoria, and Tasmania, spanning 16 degrees of latitude. The primary purpose of this study was to explore factors and mechanisms underlying MS development. A total of 282 incident cases with the first diagnosis of a demyelinating event were recruited into the study from 1 November 2003 to 31 December 2006. Extensive individual information and health history that occurred prior to symptom onset was obtained for each participant, and this dataset was used to analyse the associations between environmental/behavioural factors and ASO.

To examine associations between established/potential beneficial/detrimental factors and the rate of MS progression, cases in the Ausimmune Study were prospectively followed for at least 5 years in the AusLong Study. The main endpoints of this study were as follows: (1) hazard of time to conversion to MS; (2) hazard of time to subsequent relapses; and (3) annualised disability progression as measured by change in the Expanded Disability Status Scale. With this prospective cohort study, we investigated the associations between EBV/HHV6 infections, SLEs, and MS clinical course.

1.4 Outline of the thesis

The focus of this thesis is to examine the effects of environmental/behavioural factors on the ASO and clinical course of MS. In Chapter 2, a brief overview of MS is given. Chapter 3 reviews the role of viral infection (EBV, HHV6, and human endogenous retrovirus) on MS development and progression. This chapter has been published in the *Journal of the Neurological Sciences*. Chapter 4 discusses the first aim of this

thesis – assessing the association between latitude and ASO. This chapter has been published in the *Journal of Neurology, Neurosurgery & Psychiatry*. Chapter 5 discusses the second aim of this thesis – the role of a range of environmental or behavioural factors on ASO. Chapter 6 discusses the third aim of this thesis – the association between baseline-measured serological and viral load measures of EBV/HHV6 infection and MS clinical course (conversion to MS, relapse, and disability progression). Chapter 7 discusses the fourth aim of this thesis–the association between SLEs and MS clinical course. Chapter 8 draws the primary conclusions of this thesis and discusses future research.

1.5 References

1. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron* 2006;52(1):61-76. doi: 10.1016/j.neuron.2006.09.011
2. Browne P, Chandraratna D, Angood C, et al. Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology* 2014;83(11):1022-4. doi: 10.1212/WNL.0000000000000768
3. Noseworthy JH, Lucchinetti C, Rodriguez M, et al. Multiple sclerosis. *The New England journal of medicine* 2000;343(13):938-52. doi: 10.1056/NEJM200009283431307
4. Palmer AJ, Colman S, O'Leary B, et al. The economic impact of multiple sclerosis in Australia in 2010. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(12):1640-6. doi: 10.1177/1352458513488230
5. Benito-Leon J, Mitchell AJ, Rivera-Navarro J, et al. Impaired health-related quality of life predicts progression of disability in multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2013;20(1):79-86. doi: 10.1111/j.1468-1331.2012.03792.x [published Online First: 2012/06/30]
6. Benito-Leon J, Rivera-Navarro J, Guerrero AL, et al. The CAREQOL-MS was a useful instrument to measure caregiver quality of life in multiple sclerosis. *Journal of clinical epidemiology* 2011;64(6):675-86. doi: 10.1016/j.jclinepi.2010.08.003 [published Online First: 2010/11/13]
7. Rivera-Navarro J, Benito-Leon J, Oreja-Guevara C, et al. Burden and health-related quality of life of Spanish caregivers of persons with multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2009;15(11):1347-55. doi: 10.1177/1352458509345917 [published Online First: 2009/10/03]
8. Mitchell AJ, Benito-Leon J, Gonzalez JM, et al. Quality of life and its assessment in multiple sclerosis: integrating physical and psychological components of wellbeing. *Lancet neurology* 2005;4(9):556-66. doi: 10.1016/s1474-

4422(05)70166-6 [published Online First: 2005/08/20]
9. Benito-Leon J, Morales JM, Rivera-Navarro J, et al. A review about the impact of multiple sclerosis on health-related quality of life. *Disability and rehabilitation* 2003;25(23):1291-303. doi: 10.1080/0963828031000160859 1 [published Online First: 2003/11/18]
10. Tao C, Simpson Jr S, Taylor BV, et al. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. *Journal of the neurological sciences* 2017;372:239-49. doi: http://dx.doi.org/10.1016/j.jns.2016.11.060
11. Ponsonby AL, Lucas RM, Dear K, et al. The physical anthropometry, lifestyle habits and blood pressure of people presenting with a first clinical demyelinating event compared to controls: the Ausimmune study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(13):1717-25. doi: 10.1177/1352458513483887
12. Munger KL, Levin LI, Hollis BW, et al. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA : the journal of the American Medical Association* 2006;296(23):2832-8. doi: 10.1001/jama.296.23.2832
13. Lucas RM, Ponsonby AL, Dear K, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011;76(6):540-8. doi: 10.1212/WNL.0b013e31820af93d
14. Butzkueven H, Chapman J, Cristiano E, et al. MSBase: an international, online registry and platform for collaborative outcomes research in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2006;12(6):769-74. [published Online First: 2007/02/01]
15. Lucas R, Ponsonby AL, McMichael A, et al. Observational analytic studies in multiple sclerosis: controlling bias through study design and conduct. The Australian Multicentre Study of Environment and Immune Function. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2007;13(7):827-39. doi: 10.1177/1352458507077174 [published Online First: 2007/09/21]

Chapter 2 Literature review of multiple sclerosis

2.1 Preface

This chapter discusses different aspects of multiple sclerosis (MS) that are important for the understanding of the subsequent chapters, including phenotypes, age of symptom onset (ASO), clinical course, risk factors, diagnosis, and treatment. This chapter does not provide a full literature overview on MS, but merely focuses on the topics of this thesis.

2.2 Clinical phenotypes of MS

2.2.1 Relapsing-onset MS

According to the pattern of disability progression after disease onset, MS can be classified into two major onset types: relapsing and progressive MS. Further subcategorisation is based on the presence/absence of disease activity. The relapsing-onset type is the most common type and characterised by multiple episodes of neurological dysfunction. Typical relapsing-onset MS consists of four stages: at-risk of MS, asymptomatic, relapsing phase, and progressive phase. Full or partial recovery from relapses during early stages of the disease constitutes the major clinical characteristic[1], incomplete recovery from severe relapses lead to the disability progression. Accumulation of intermittently focal inflammation over time contribute to the progression of disability in people with MS. Approximately 85% of relapsing-remitting MS (RRMS) patients will develop secondary-progressive MS (SPMS) within 10 or 20 years after disease onset, which is characterised by the relentlessly progressive accumulation of disability with or without superimposed relapses. It is currently recognised that the process of progression to SPMS is gradual[1], where the

frequency of relapses decreases[2 3] and shifts to a sustained deterioration of neurological function.

2.2.2 Progressive-onset MS

According to the absence/presence of disease activity (clinical relapses and MRI-identified lesions) in patients with progressive MS, clinical phenotypes of progressive MS can be categorised into active and non-active, respectively[1]. This classification includes inflammatory lesion activity and gradual worsening.

Notably, pathological mechanisms underlying progressive-onset MS are quantitatively different from RRMS. Focal new and active lesions in the white matter tissue are common in the early stages, but become rare when patients enter the progressive phase[4]. Cortical demyelination increases massively when patients suffer from a progressive phase, and diffuse changes in the normal-appearing white matter also become pronounced[5].Focal lesions in RRMS patients tend to arise from regions with high venous density, while in patients with the progressive phase, lesions tend to locate at regions with a lower venous density[6]. A higher perfusion may aid the lesions in patients with RRMS in recovering more complete, but in patients who are in the progressive phase, lesions may become permanent due to the lack of perfusion. Over the course of disease, diffuse microglial activation and neurodegeneration become more prominent in normal-appearing white and grey matter[7]. Failure of remyelination cause axonal degeneration and disease progression.

2.2.3 Paediatric-onset and late-onset MS

Paediatric-onset MS (POMS) is commonly defined as patients with the onset of MS prior to the age of 18 years. Similar to patients with adult-onset MS (AOMS), EBV infection[8-10] and *HLA-DR15* genotype[11] have been associated with an increased risk of MS in paediatric patients. More than 98% of paediatric MS manifests as relapsing-onset form, so progressive-onset disease in paediatric MS patients should be diagnosed carefully with extensive assessment for alternative diseases[12]. Compared to AOMS patients, rate of relapses tends to be higher in patients with POMS. Relapses in POMS tended to be more severe than AOMS, but recovery was also more complete[13 14]. Although patients with POMS take a longer period to convert to SPMS, they still tend to have an earlier age at onset of SPMS (41 years for patients with paediatric MS vs 52 years for adult)[15]. Incomplete recovery from the first attack has been associated with a shorter time until reaching an EDSS 4 (13.5 years in those with residual disability vs. 25.4 years in those with complete recovery from the first attack)[15]. Like AOMS, a female preponderance has been found in patients with POMS as well, with female:male ratio up to 4.5:1[12]. Notably, for those with an extremely early onset (<12 years of age), the sex ratio could be is close to 1:1. The marked increase in the proportion of female patients after 12 years of age is thought to be driven by change in hormonal state[16]. The initial clinical manifestation of paediatric MS patients is highly variable, but the attack tends to be monofocal and localise to one area of the CNS in up to 90% paediatric patients[17]. Treatment for POMS is similar to AOMS, and interferon-β was commonly treated as first-linetreatment[18 19]. An earlier application of DMTs has also been shown to be beneficial for paediatric MS patients[20].

In most studies, it has been found that 1.4%-9.9% of all patients have late-onset MS (LOMS), defined as symptoms onset at 50 years of age or later[21]. A female preponderance is also found in patients with LOMS, with a similar sex ratio to adult onset MS[22]. Initial presentation in LOMS is commonly monosymptomatic, with motor and cerebellar dysfunction[23]. Like patients with adult-onset MS, progressive-onset type is the most common clinical course in LOMS[24]. LOMS is associated with a shorter period before reaching EDSS 6.0[25 26].

In summary, with increasing of age at onset (POMS-AOMS-LOMS), the proportions of patients with progressive-onset forms increased, disability progression is accelerated, and female comprise an increasing proportion of cases.

2.3 Age of symptom onset

The primary outcome of chapters 4 and 5 is ASO, the age of the first demyelinating event, we will discuss the clinical significance of ASO here. Associations between environmental/genetic factors and ASO will be discussed separately when the effects of each factor are introduced. A timely diagnosis of MS is difficult for patients with atypical symptoms, which may lead to the inaccurate measurement of ASO. In the AusLong study, all patients were carefully reviewed, especially those with a previously undiagnosed neurological event thought to be demyelination. Also, for patients with progressive-onset MS, an accurate measure of ASO was extremely difficult, given the more insidious development of symptoms over time. Therefore, in Chapter 5, a sensitivity analysis was performed that was restricted to patients with relapsing-onset MS to avoid the potential measurement error. However, in Chapter 4, ASO was measured by different neurologists in different clinical centres, potential

measurement error of ASO was hard to avoid and may influence the validity of the results.

2.3.1 Associations between ASO and long-term prognosis

Associations between ASO and consequent disease progression are complicated: an earlier onset is associated with a slower progression, whereas a later onset is associated with an accelerated progression, the age of reaching severe disability or progressive-MS is similar[26-29]. Although it has been recognised that late onset is associated with a more aggressive clinical course and can lead to severe disability sooner, delaying disease onset is still of great importance for patients with MS. Mounting evidence has confirmed that an earlier onset could increase the risk of conversion from clinically isolated syndrome (CIS) to MS[30-34]. Following 114 patients with acute partial transverse myelopathy, Ruet et al[30] found that patients with a younger age had a significantly higher odds of conversion to MS (OR 7.82, $p<0.001$). The authors controlled for some covariates in the multivariable analysis. The strong magnitude supported the validity of the result, however, some limitations influenced the generalisation: 1) all patients were recruited from one hospital and were not population-based which could have led to selection bias; and 2) only patients with acute partial transverse myelopathy were recruited, which led to spectrum bias. Therefore, the results in this study were not representative of the MS population. Recruiting CIS patients from 33 centres in 17 countries, one large prospective cohort study with 623 CIS patients demonstrated that an earlier onset was associated with a higher hazard of conversion to MS [hazard ratio $(HR)_{per\ year=}0.98$, 95% confidence interval (CI) = 0.98–0.99, $p<0.001$][31]. The lack of standardised protocols and not controlling for the difference between study centres influenced the interpretation of

the results. However, the large sample size and controlling for confounder bias supported the significant association between age and conversion to MS. Following 1015 CIS patients prospectively for approximately 7 years, Tintore et al[33] reported that cases with an earlier onset had a higher hazard of conversion to MS: when compared with those with a late onset (>40 years), the HR of those aged 30 to 39 years, 20 to 29 years, and 19 years or less were 1.4, 1.8, and 1.9, respectively. Patients were recruited from 2005 to 2013, so birth cohort effects may influence the results, but the prospective cohort design and large sample size supported the detrimental effects of younger age on conversion to MS. Results from the MSBase registry[34] also supported the significant association between older onset and decreased hazard of conversion to MS (HR 0.91, 95% CI = 0.89-0.93; p<0.001). In brief, consistent results from a number of studies supported the association between earlier onset and higher hazard of conversion to MS.

2.4 Conversion to MS, relapses, and disability progression

The outcome of chapters 6 and 7 is MS clinical course, which includes conversion to MS, relapses, and disability progression. Conversion to MS and relapses represent the disease activity, whereas disability progression represents a sustained accumulation of neurological impairment. The diagnostic criteria used for conversion to MS and relapses in MSBase and the Ausimmune Study is the McDonald criteria[35 36], which will be discussed in detail later. Quantification of the degree of disability due to MS is commonly measured with the Disability Status Scale (DSS, 10-point scale)[37] or the Expanded Disability Status Scale (EDSS, 10-point scale with half-step increments (not between 0-1)[38]. A lower score (<4) of EDSS is measured with neurological

examination of eight functional systems, while the higher score is predominantly based on walking ability (>4) or handicaps (>6).

Several studies have demonstrated that the intra-rater agreement is slightly higher than the inter-rater agreement, and the reliability is significantly lower when the EDSS score is less than 4[38-40]. Since most patients in AusLong study were still at the benign stage, the reliability of EDSS might be inaccurate. EDSS at baseline interview and 5-year review in Ausimmune and AusLong study were measured with an experienced neurologist in each study centre, and all EDSS was reviewed thereafter to ensure the reliability. No evidence suggested that this potential measurement error of EDSS would be differential by the exposures of interest, and we believed this potential measurement error would not impact the results materially.

An advantage of the EDSS is the comparability of the scores between different studies, and the measurement is robust during a long period of follow-up. It is acknowledged that the increase of EDSS is not linear, however, the AusLong study participants were people with a first clinical diagnosis of demyelination at study entry and 87.6% had an EDSS score ≤ 4 at the 5-year review. Previous research has suggested that the disability progression is comparative linear when EDSS was ≤ 4. Time to particular EDSS hallmarks were less suitable as the participants were not assessed frequently enough by neurologists to reliably determine the date participants reached an EDSS of three or four. Therefore, annualised change of EDSS in the AusLong study was the best measure of disability progression.

All relapses in AusLong study were diagnosed with a standardised protocol by an experienced neurologist in each study centre. Moreover, every relapse was reviewed

thereafter to ensure the validity of the diagnosis. However, due to the nature of MS, some minor attacks are extremely difficult to detect. It would be difficult to predict whether these minor attacks affected our main associations of interest and in which direction.

Time to relapse rather than annualised relapse rates was used in this thesis, which increased the temporal precision, as all information was included about the clinical course (dates of relapses, rather than just their number) and the fluctuation of status over time. While relapses of the same person were clustered, multiple relapses were regarded as independent observations on the assumption that time until a prior event does not affect the composition of the risk set for a subsequent event. Cox proportional hazards models were used to include the information of repeated events[39]. Also, some research has used this model to analyse the association between exposures and relapses[40 41].

Here we will discuss the relationship between conversion to MS, relapses, and disability progression, and their associations with MRI parameters. Associations between environmental or behavioural factors and MS clinical course will be discussed next when each risk factor is introduced.

2.4.1 Conversion to MS

Using clinical and biochemical variables that are assessable at baseline to predict the risk of conversion from CIS to MS is always a complex puzzle in clinical practice. Current evidence indicates that whether or when a patient with CIS will convert to MS is predictable to some extent. A number of factors have been assumed to contribute to increased risk, and among them, higher load of lesions on MRI is the

strongest predictive factor in MS prognostic study[30 31 33 34 42-45]. Current diagnostic criteria refer to patients with disseminated lesions on MRI, but without repeated clinical attacks, as possible MS[35]. A retrospective cohort study[42] with 137 CIS patients (median time of follow-up: 3.1 years) reported that a higher load of MRI lesions (HR 1.67, 95% CI: 1.1–2.6, p=0.028) at baseline was associated with an earlier conversion to MS. Following 220 CIS patients (receiving interferon-β) prospectively for 2 years, Kalincik et al[45] found that more than 1% decrease in the corpus callosum cross-sectional area between baseline interview and 6 months after CIS (HR 2.5, 95% CI: 1.5-4) and an increased volume of T2 lesions (HR 1.8, 95% CI: 1.1-2.9) were associated with a higher hazard of conversion to MS. A multicentre and retrospective study[43] of 1165 CIS patients found that increased lesion load at baseline (7.6 ± 8.1 cm^3 vs. 4.6 ± 6.7 cm^3, $p<0.001$) was associated with an increased risk of conversion to MS. Current studies have confirmed the clinical significance of a high load of MRI lesions in MS prognosis.

Mounting research has indicated that the presence of oligoclonal bands in cerebrospinal fluid (CSF) is associated with a higher hazard of conversion to MS. One recent natural history study[33] with 1015 CIS patients found that presence of oligoclonal bands increased the hazard (HR 1.3, 95% CI: 1.0-1.8) of conversion to MS. A similar magnitude was reported from the MSBase registry (HR 1.40, 95% CI: 1.11-1.77; p=0.004)[34]. Converging evidence supports that the presence of oligoclonal bands should be regarded as a medium prognostic factor for conversion to MS.

2.4.2 Relapse

More than 85% of patients with MS experience relapses during the disease course[46]. The relapse rate varies between 0.3 and 1.0[47]; however, this may slightly underestimate the actual rate, owing to a proportion of patients who do not identify/report their attacks[48]. The relapse rate is highest in patients with MS aged less than 30 years [odds ratio (OR): 1.42, $p<0.001$][49]. The severity of relapses increases with age, and the ability to recover from them decreases[50].

A number of long-term follow-up therapeutic studies[51-56] supported that relapses could predict disability in the long run; however, potential influences of therapy and the lack of association between pre-study relapses and disability[56] weakened the strength of the relationship. Natural history studies have suggested that only relapses that occurred early in the disease course are associated with long-term disability (reviewed by Goodin[57]). With the analysis of 806 relapsing-onset patients from an original population-based natural history cohort, Scalfariet al[3] discovered that only relapses that occurred within 2 years after disease onset could predict the long-term severity of disability, whereas relapses occurring beyond 2 years showed no relationship with disability levels. Disability level was measured with the DSS, and time to DSS was analysed with survival analysis in this study. Although some important confounders were not controlled in this study, the elegant study design and appropriate statistical method still supported the validity and generalisation of the results. With the same cohort, Scalfari et al[58] also found that a shorter interval between the first and second attack was associated with a faster progression of disability. Following 1609 relapsing-onset CDMS patients for about 13 years, Emmanuelle et al[59] found that the number of relapses during the first 2 years were

significantly correlated with disability progression, as time to irreversible DSS 3 was 12.2 years in those with only 1 relapse in the first two years, while the duration decreased to 7.7 years if patients experienced 2 or more relapses. However, number of relapses during the first two years was not associated with the duration from DSS 3 to DSS 6, which suggested the hypothesis that disability progression in patients with MS follows a two stage process, from disease onset to irreversible DSS 3 and from DSS 3 to DSS 6. The results were supported by the assumption[60] that focal inflammation was responsible for the disease at early stage while diffuse inflammation and neurodegeneration was responsible for the late stage. In this study, all patients were selected from a regional referral centre and diagnosed with Poser's criteria. Strengths of this study were the prospective cohort study design and the consideration of important confounders.

Interestingly, male patients have shown a faster progression of disability despite a lower frequency of relapses compared to female patients[61 62]. The difference of clinical symptoms between male and female patients may contribute to this paradox. Female patients tend to present with sensory and visual symptoms while male patients tend to present with brainstem, pyramidal and cerebellar symptoms[50]. An improved recovery has been observed for those with visual, sensory and brainstem symptoms[50].

Most studies in the literature supported a high correlation between MRI lesions and clinical relapses in patients with MS, owing to the quantitative relation of treatment effects detected by MRI parameters and relapse rates from randomised controlled trial (RCT) studies[63-68]. A number of clinical trials have used MRI parameters as a surrogate for clinical relapses.

2.4.3 Disability

A number of RCT studies[65-67] have supported the high correlation between treatment effects on MRI lesions and disability progression. Mounting studies[67] have suggested that brain atrophy and lesion volumes could predict the long-term clinical evolution in patients with MS. A retrospective study[69] including 261 MS patients with a follow-up period of 10 years found that central atrophy and lesion volume change within 2 years after disease diagnosis were associated with EDSS at 10 years: the correlation was 0.74, controlling for imaging protocol, baseline EDSS, and disease modifying therapy (DMT). This study was designed retrospectively and patients were recruited from 8 centres. The correct temporality of association (exposure before outcome) and application of standardised protocols supported the conclusion that changes of MRI signs at early stages of disease may predict the subsequent disability progression. Following 107 CIS patients (recruited from 1984 to 1987) for approximately 20 years, Fisniku et al[70] found that change of lesion volume at the early stage of disease (0-5 years) was associated with the level of disability after 20 years ($r=0.48$, $p<0.001$). Uher et al[71] conducted a prospective cohort study of 220 CIS patients, and during the 48 months of follow-up, they found that increased lateral ventricle volume ($p<0.001$), decreased grey matter ($p=0.011$), and decreased cortical volume ($p=0.001$) were associated with a sustained disability progression. A prospective cohort study[52] following 163 CIS patients for 15 years treated with interferon-β showed that enhancing lesions (OR: 8.96, $p<0.001$) were associated with worsening disability progression. Several limitations such as the initial RCT design, the fact that all patients were administered with interferon, and that EDSS scores being self-reported influenced the validity and generalisability of the results. In summary, several

prospective cohort studies supported that MRI changes at the early stages of disease could predict the long-term disability progression in patients with MS.

Disability levels also showed a close relationship with psychological dysfunction. With 1040 patients, Ruano et al[72] showed that a higher level of EDSS was associated with the presence of cognitive impairment (OR: 1.80, 95% CI: 1.51-2.15; $p<0.001$). Another cross-sectional study[73] with 253 MS patients also supported this conclusion. Following 260 MS patients after the participation of a RCT study, Goodin et al[56] reported that disability at disease onset was significantly correlated with cognitive impairment ($r=0.12$, $p<0.001$) after approximately 16 years. However, making use of recruitment from RCTs can lead to biased recruitment and generalisability issues to the broader population with MS, while not controlling for possible confounders limits the interpretation of results.

2.5 Associations between smoking, UVR/25(OH)D, offspring number, stressful life events, genetic factors, and MS clinical course

2.5.1 Tobacco smoking

Tobacco use was more common in patients with MS than in the general populations[74], with approximately half of MS patients having smoked tobacco at some time[75]. Tobacco smoking is viewed as an established risk factor for MS, due to the consistent associations and the evidence from good-quality cohort studies[76-79].

Using the Hill criteria for causation, one recent meta-analysis[18] including 23 studies demonstrated strong evidence for a causal role of tobacco smoking on MS development, with 6 criteria (consistency, temporality, biological gradient, plausibility, and coherence) being satisfied and only one criteria was modestly

satisfied (strength, OR/RR 1.54, 95% CI 1.46-1.63). The interesting and superficially counterintuitive phenomena wherein the prevalence of multiple sclerosis has increased while that of smoking has decreased may be the result of a number of factors: 1) the implementation of MRI and the application of McDonald's criteria allowed an earlier and more accurate diagnosis of MS, 2) the onset of MS was driven by the interaction of many factors, of which smoking is but one, the population attributable fraction of smoking was around 40%. Indeed, not all MS patients need to have a history of smoking, since smoking was only one underlying cause in a proportion of patients. Rothman's "causal pie model" is an useful framework to understand this phenomenon: tobacco smoking is an event that plays a necessary role in the onset of MS in some people, however, some persons can get MS through other pathways (such as EBV infection, low vitamin D levels, and *HLA-DR15*1501* genotype).

Mechanisms underlying the association between tobacco smoking and MS natural history are complex, with direct effects of tobacco smoking on detrimental modulation of humoral and cell-mediated immunity[81], acceleration of axonal degeneration[82], and increased concentration of cyanide intoxication in serum that could cause the wide demyelination[83 84]. In addition, associated comorbidities such as increased risk of respiratory infections may also be involved[85].

The risk of MS in smokers is approximately 50% higher than in non-smokers[86]. Among all environmental risk factors [smoking, low actinic damage, history of infectious mononucleosis (IM), and low 25-hydroxy vitamin D at MS onset], the population attributable fraction of tobacco smoking was 35.1%[87]. Current research has

suggested that passive smoke exposure could also increase the risk of MS[88-90]. A large case-control study[90] with 695 incident MS cases and 1635 control subjects who had never smoked found that MS cases had a higher exposure to passive smoking (OR 1.3, 95% CI: 1.1-1.6), and the effects were stronger when the duration of passive smoking increased (test for trend p=0.003). With the assessment of cotinine levels in serum (which is an objective measurement to reflect the recent amount of tobacco use[91]), two studies[79 92] both found that elevated cotinine levels were associated with increased risk of MS.

The association between tobacco smoking and MS clinical course is not as strong as for MS onset, but there is a fair amount of evidence supporting that smoking may also have adverse effects on the clinical course of MS. Table 1 shows some key studies about tobacco smoking and its effect on MS clinical course. A prospective cohort study following 129 CIS patients for 3 years found that cases with a history of smoking (smokers vs. non-smokers) at baseline had a higher hazard of conversion to MS (HR: 1.83, 95% CI: 1.17-2.84, p=0.008) than non-smokers, and the association did not alter much after controlling for sex, age at onset, interferon-β use, and number of T2 lesions on MRI[93]. This study also found that the EDSS score was not different between smokers and non-smokers (p=0.90) after 3 years. Recruitment of all patients was in single clinical centre from 1994 to 2004, the long period of recruitment and not being a population-based study design does negatively influenced the external validity of the findings. Two other studies[94 95] found a significant association between tobacco smoking and disability progression as measured by Multiple Sclerosis Severity Score (MSSS) or time to EDSS. Some large prospective cohort studies showed that smoking status was associated with numbers and volumes of contrast enhancing lesions[96], and

with whole brain volume[97]. Although selection bias[93], birth cohort effect[93 96], spectrum bias[72] and insufficient time of follow-up[96] could have influenced the validity of some of these studies, this combined research suggests that tobacco smoking has a detrimental effect on MS clinical course.

Most studies[98-101]also supported a detrimental effect of tobacco smoking on subsequent progression from RRMS to SPMS. Following 179 RRMS patients for 5 years, Hernan and colleagues[98] found a higher hazard (HR: 3.6, 95% CI: 1.3-9.9) of progression to SPMS when comparing smokers with non-smokers. This was a prospective study with patients selected from a database with more than 3 million Britons, and all patients were diagnosed using the Poser's criteria, and age, sex, and initial symptoms were controlled for in the multivariable analysis. Healy and colleagues conducted a similar study[99] following 891 patients for approximately 3 years, and found a higher hazard of progression to SPMS (HR: 2.50, 95% CI: 1.42-4.41) when comparing current smokers to never-smokers, but no association was seen when comparing ex-smokers with never-smokers (HR: 1.05, 95% CI: 0.59-1.84).

Table 2.1. Key studies assessing association between tobacco smoking and MS clinical course

First Author (year)	**Research design**	**Main findings**
Di Pauli (2008)[93]	prospective cohort study, 129 CIS patients for 36 months	A history of smoking at baseline was associated with a higher hazard of conversion to MS [HR: 1.8 (1.2-2.8), p=0.008].
Pittas (2009)[94]	prospective cohort study, 203 MS patients for 3 years	Cumulative pack-years smoked after study entry was associated with higher MSSS.
D'Hooghe (2012)[95]	cross-sectional study, 1372 MS patients	In RRMS, cigarette smoking was associated with a higher risk for reaching EDSS 6 (p<0.001)
Horakova (2013)[96]	prospective cohort study, 211 CIS patients for 24 months	Active smoking status was associated with increased number and volume of T1 Gd+ but not with relapses, conversion to MS number, T2 lesions, and brain atrophy.
Munger (2015)[102]	prospective cohort study, 468 CIS patients for 5 years	No association between cotinine levels and conversion to MS, risk of relapses, and MRI measures.

Kvistad (2016)[103]	prospective cohort study, 87 RRMS patients for 2 years	No association between cotinine levels and new T1 Gd+ lesions [OR: 0.76 (0.39-1.50), p=0.43; new T2 lesions [OR: 0.87 (0.43-1.75), p=0.69]; number of relapses (p=0.41); disability progression (p=0.14).
Hernan (2005)[98]	prospective cohort study, 179 RRMS patients for 5 years	Smokers had a higher hazard of progression to SPMS (HR: 3.6, 95% CI: 1.3-9.9).
Koch (2007)[101]	Cross-sectional study, 364 MS patients	No association between smoking behaviours and progression to SPMS and disability progression.

CIS, clinically isolated syndrome; HR, hazard ratio; MS, multiple sclerosis; RRMS, relapsing-remitting MS; OR, odds ratio; CI, confidence interval; SPMS, secondary progressive MS.

2.5.2 UVR exposure/25(OH)D level

Positive associations between latitude of residence and incidence[104 105] and prevalence[106] in MS patients have been among the most striking findings in MS epidemiological studies. This latitudinal gradient may arise from a range of factors: social-economic factors, EBV infection and ultraviolet radiation. One pooled analysis[107] showed the significant association between latitude of residence and positivity of EBV infection in patients with MS. Another meta-analysis[108] with more than 40,000 participants from northern hemisphere, found that patients with MS were more likely to be born in May and less likely to be born in November, which suggests that seasonality may be a risk factor. The variation of ultraviolet radiation levels may contribute substantially to this latitudinal gradient[109]. With data of 54 studies across Canada, Sloka et al[110] found that UVB intensity had a closer relationship (about 20-fold) with the geographic distribution of patients with MS than latitude, which suggests that the latitudinal gradient could be mostly driven by UVB intensity.

Converging evidence supports the hypothesis that insufficient outdoor activity during childhood and adolescence are associated with an elevated risk of MS[114-118]. A case-control study[114] in Tasmania of 136 MS patients and 272 healthy control subjects found that sufficient summer sun exposure (> 2-3 h/day during weekends and

holidays) during childhood and adolescence was associated with a decreased risk of MS (OR: 0.31, 95% CI: 0.16-0.59) in a dose-dependent manner. Using objective measurement of sun exposure, this study also found a significant association between higher actinic damage and lower MS risk (OR: 0.32, 95% CI: 0.11-0.88). In line with these results, a subsequent multinational case-control study[115] including 1,660 MS patients and 3,550 control subjects found that insufficient sun exposure in summer was associated with an increased risk of MS, and this association was strongest when sun exposure was measured during adolescence. A large multi-centre study[119] in Australia including 1,524 MS cases found that low maternal exposure to UVR in the first trimester was associated with a higher risk of MS in the offspring [highest category vs. lowest category, incidence rate ratio (IRR): 1.67, 95% CI: 1.18-2.37]. This study also found that the association between month of birth and MS onset was totally driven by insufficient exposure to UVR. Possible mechanisms underlying the association include important role of first trimester vitamin D concentrations in the development of the CNS[120] and immune tolerance[121].

For most individuals, skin exposure to UVR is the primary source of vitamin D[116], and many have believed that the effect of sun exposure on MS was through its effect on vitamin D levels[122]. However, UVR has additional effects on the body, independent of vitamin D. Indeed, findings in animal models[123] have suggested that the protective effect of UVR on MS pathology are independent of the status of 25-hydroxyvitamin D [25(OH)D], because observations from experimental autoimmune encephalomyelitis (EAE) found that continuous exposure to UVR could suppress the clinical signs of MS, and this process occurred independent the elevation of 25(OH)D levels in serum. In agreement with these animal models, the Ausimmune Study[117]

[216 first demyelinating event (FDE) cases and 395 healthy controls] found that insufficient sun exposure and low vitamin D levels were two independent risk factors for FDE development (OR: 0.39, 95% CI: 0.17-0.92; OR: 0.93, 95% CI: 0.86-1.00, respectively) in a multivariable model.

Vitamin D is a steroid hormone, and the pleiotropic effects have been demonstrated, as well as its effect on immune system modulation in MS pathology[124 125]. There are two forms of vitamin D: cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). After metabolism in the liver, 25(OH)D is synthesised, and it is this form that is used to measure vitamin D levels in serum[126]. Through the regulation of local and systemic immune systems, low 25(OH)D levels have been regarded as an established risk factor for MS development (reviewed by van der Mei[127]).

Genetic studies also support that vitamin D levels can modulate the susceptibility to MS. The enzyme 25(OH)D-1a-hydroxylase, which is needed for vitamin D activation, is encoded by the *CYP27B1* gene. Ramagopalan et al[128] found that mutations in the *CYP27B1* gene were associated with an increased risk of MS. A vitamin D response element has been found in the promoter region in *HLA-DR15,* which suggests that an interaction between vitamin D and *HLA-DR15* may modulate the onset of MS[129]. Some other genes associated with vitamin D metabolism were also found to contain single nucleotide polymorphisms (SNPs) related with MS onset[130 131].All these studies provide additional support in favour of a role for vitamin D levels in the determination of MS risk.

As previously discussed, not all MS patients need to be vitamin D insufficient, though this can be a component leading to MS. The Bradford-Hill criteria could be used to

support the association between insufficient levels of vitamin D and MS onset: 1) Strength: The strength of the association between decreased vitamin D levels and MS onset was strong[19]. In a nested case-control study with 192 MS patients with blood samples collected prior to disease onset and 384 controls, the authors found that higher levels of Vitamin D was associated with a decreased risk of MS (OR 0.39, 95% CI 0.16-0.98); 2) Consistency: The association between vitamin D insufficiency and MS onset was consistent across multiple study settings and in different populations[20]; 3) Specificity: In contemporary epidemiological view, specificity dose not mean that insufficient levels of 25(OH)D could only cause MS but no other diseases. We should view specificity more generally, the effects of immunological regulation allows vitamin D to be relevant to many other diseases. However, we should notice that vitamin D was not associated with diseases randomly: it was associated with MS but not many other neurological diseases such as schizophrenia or brain cancer; 4) Temporality: Prospective cohort studies have demonstrated that insufficient vitamin D and related parameters like vitamin D intake and historical sun exposure prior to MS onset were associated with subsequent MS risk and clinical course[19]; 5) Biological gradient: Evidence supports a dose–response relationship between vitamin D levels and MS risk arises from a range of cohort and case-control studies. Studies collecting serum 25(OH)D before and at the time of MS onset both supported a dose–response relationship between vitamin D insufficiency and MS risk. One prospective cohort study with 25(OH)D collecting prior to MS onset showed that every 50 nmol/l increase in 25(OH)D was associated with a decreased odds of MS onset (OR 0.59 ,95% CI 0.36-0.97)[19]; 6) Biological plausibility: vitamin D encourages a more efficient and specific immune response, both adaptive and innate, and

encourages an anti-inflammatory and more tolerogenic state. Given MS is an autoimmune disease, these effects line up with the observed inverse associations between 25(OH)D and MS; 7) Coherence: studies in humans and animal models and cell culture line up, including the aforementioned studies of UV in EAE all supported a protective effect of higher 25(OH)D levels on MS onset; 8) Experiment: The criteria of experiment is somewhat difficult to be satisfied, as it is an essential nutrient for the general populations and it is obviously unethical to withhold vitamin D from the placebo group. Thus, current experimental evidence mostly comes from the effect of vitamin D supplementation in decreasing of disease activity. Including 49 MS patients, an unblinded randomized trial with a follow-up of 52 weeks found that treatment group patients had a reduction of T-cell proliferation and fewer relapses[21]; 9) Analogy: The causal roles of vitamin D have been supported by Hill's criteria in several other diseases, such as overall cancer risk[22], breast cancer risk[23] and cardiovascular disease risk[24]. Thus, the analogy criterion was met.

Table 2 shows some key studies assessing the association between 25(OH)D levels and MS clinical course. With the prospective cohort design of 145 RRMS patients, our group[135] found that higher 25(OH)D levels were associated with reduced hazard of relapses (HR: 0.90, p=0.016). All patients in this study were recruited in southern Tasmania from 2002 to 2005 and diagnosed using the 2001 McDonald criteria. This, along with the prospective cohort design, repeated measurement of 25(OH)D and sophisticated statistical methods provide strong support for the association between levels of 25(OH)D and relapses. Another prospective study[136] with 469 MS patients found a significant association between 25(OH)D levels and MRI activity and disability progression, but found no association between vitamin D levels and risk of

relapses. As only white participants with relapsing-onset MS were included in this study, results in this study may not be generalisable to the entire population. However, repeated measurement of 25(OH)D and prospective study designs still supported the beneficial effects of 25(OH)D in MS disease activity. Martinelli el al[137] conducted a prospective study of 100 CIS patients and found that decreased levels of baseline 25(OH)D were associated with an elevated hazard of conversion to MS in a dose-dependent manner. With a prospective design of 178 MS patients, Stewart and colleagues[138] found a significant interaction between 25(OH) levels and interferon-β on hazard of relapses ($p_{interaction}$=0.003), with association of elevated 25(OH)D and lower hazard of relapses being found significant in patients with interferon-β administration. Other prospective cohort studies further supported the protective role of high 25(OH)D levels on MS clinical course[139-141]. In conclusion, a number of high-quality prospective cohort studies supported the beneficial effects of increased 25(OH)D on MS disease activity. However, the effects of 25(OH)D on long-term disability progression have been less definitely established.

A number of prospective cohort study have shown that patients with MS had a decreased level of 25(OH)D since adolescence, the temporal relationship with exposures prior to outcomes indicated that the possibility that 25(OH)D is a negative acute phase reactant is low. Given the protective associations of vitamin D with MS onset and progression, it was hypothesised that vitamin D supplementation can reduce MS disease activity[142-145]. Soilu-Hanninen and colleagues conducted a randomised trial including 66 RRMS patients using IFNB-1b use, showing no significant association between vitamin D supplementation and T2 burden of disease (p=0.105), new T2 lesions (p=0.29), and disability accumulation (p=0.07)[142]. A subsequent meta-

analysis[143] including 5 RCT studies also showed that vitamin D supplementation did not reduce the odds of relapse (OR: 0.98, 95% CI: 0.45-2.16). Although current evidence from RCTs does not support therapeutic effects of vitamin D on MS development, firm conclusions are not yet possible due to limitations such as small sample sizes, heterogeneity of administered dosing, and distinct outcome measures in the current literature.

Table 2.2 Key studies assessing associations between vitamin D and MS clinical course

First author (year)	Research design	Main findings
Simpson (2010)[135]	prospective study 145 MS patients	Inverse association between deseasonalised vitamin D and relapse (HR: 0.90, p=0.016)
Mowry (2012)[136]	prospective study 469 MS patients	Association between each 10 ng/mL increase in higher 25(OH)D and: Risk of a new T2 lesion (IRR: 0.85, 95% CI: 0.76-0.95, p=0.004); Risk of a gadolinium-enhancing lesion (IRR: 0.68, 95% CI: 0.53-0.87, p=0.002); Subsequent EDSS (β: -0.047, 95% CI: -0.091to -0.003, p=0.037); Relapse risk (IRR: 0.94, 95% CI: 0.86-1.02, p=0.12)
Martinelli (2014)[137]	prospective study 100 CIS patients	Association between 25(OH)D levels and conversion to MS: Patients with very low (< 10th percentile) (HR: 3.34, 95% CI: 1.32-8.45) Patients with low (< 25th percentile) (HR: 2.04, 95% CI: 0.96-4.36)
Stewart (2012)[138]	prospective study 178 MS patients	Association between 25(OH)D and relapse risk: IFN-β treated group (HR: 0.75, 95% CI: 0.66-0.84) Non-IFN-β treated group (HR: 0.97, 95% CI: 0.84-1.12); P for interaction 0.003
Pierrot-Deseilligny (2012)[139]	prospective study 156 patients	Every 10 nmol/L increase in 25(OH)D was associated with a 13.7% (p<0.001) reduction in incidence of relapse
Runia (2012)[140]	prospective study 73 MS patients	Higher 25(OH)D levels were associated with a decreased relapse risk (p for trend = 0.007)
Ascherio (2014)[141]	prospective study 465 MS patients	Each 20-ng/mL increment in 25(OH)D levels was associated with: 57% lower rate of new active lesions (P < 0.001) 57% lower relapse rate (P=0.03) 25% lower yearly increase in T2 lesion volume (P <0 .001) 0.41% lower yearly loss in brain volume (P=0.07) 25(OH)D ⩾50 nmol/L was associated with a decreased accumulation of EDSS (β: -0.17; P=0.004)

Soilu-Hanninen (2012)[142]	RCT 66 RRMS	Patients in the vitamin D group showed: Less increase in T2 lesion volume (median change 83 mm^3 vs. 287 mm^3, p=0.105) Lower number of new/enlarging T2 lesions (p=0.286) Less decrease in number of T1 enhancing lesions (p=0.004) Reduced disability accumulation (p=0.071) Improved timed tandem walk (p=0.076) No effect on time to first relapse (HR: 1.12, 95% CI: 0.41-3.1).
James (2013)[143]	meta-analysis 5 papers.	No association between high-dose vitamin D supplementation and risk of relapse OR: 0.98, 95% CI: 0.45-2.16
Laursen (2016)[144]	prospective study 170 MS patients	After vitamin D supplementation, each 1 nmol/L increase in 25(OH)D was associated with −0.014 (95% CI: -0.026 to -0.003, p=0.02) decrease in ARR

IRR, incidence rate ratio; ARR, annualised relapse rate; OR, odds ratio; HR, hazard ratio; CI, confidence interval

2.5.3 Offspring number

The sex ratio (female/male) of relapsing-onset MS patients increased from 2:1 to 3:1 during the last two decades, and the continuously increasing incidence has been mostly driven by females[146 147]. Increasing sex ratio by year of birth was demonstrated by one longitudinal population-based study in Canada ($r=0.84$, $p<0.001$)[148]. Women of childbearing age are more susceptible to MS, which led to the hypothesis that reproductive factors—especially pregnancy—are associated with MS.

The relationship between pregnancy and MS has been debated over the past 50 years. Relapse rate decreases significantly during pregnancy, especially in the third trimester, when compared to relapse rate prior to pregnancy; however, in the first 3 months postpartum, the relapse rate increases significantly ($p<0.001$), and then returned to the rates before pregnancy[149-152]. Decreases of relapse rate during pregnancy have been hypothesised to result from the increase of oestrogens levels. Previous *in vivo* research has found that oestrogen could modulate the profile of T helper (Th) cells, increasing levels of Th2 cells (which produce anti-inflammatory cytokines) and decreasing levels of Th1 cells (which produce pro-inflammatory cytokines)[153].

However, the relationship between pregnancy prior to MS onset and risk of subsequent MS development is still a matter of controversy. A population-based national study[154] of 4.4 million people who were born before 1989, and offspring number prior to MS onset, found that 6332 women and 3426 men were diagnosed with MS. The risk of MS for women with at least one child decreased approximately 24% when compared with nulliparous women. With additional children, the risk of MS decreased further (RR: 0.87, 95% CI: 0.71-0.82). A similar association was also seen in men: cases with no less than one child before onset had a lower risk of MS when compared with men who had no children (RR: 0.89, 95% CI: 0.80-0.98). This study suggested that biological factors were not responsible for the protective role of pregnancy on MS onset. However, a case-control study[155] conducted by our group found that giving birth to a child before onset was solely associated with reduced MS risk in women, whereas no association between offspring number and MS risk was seen in men. More well-powered prospective cohort studies are needed to confirm the link between pregnancy/offspring prior to MS onset and risk of MS development, especially whether higher offspring numbers confer a protective effect on men, and if so, the underlying mechanisms are definitely worth exploring.

The long-term effects of pregnancy and childbirth on MS clinical course are not clearly defined. A retrospective cohort study[156] including 675 relapsing-onset and 298 progressive-onset patients found that a higher number of pregnancies was associated with lower hazard of sustained EDSS 6 (two or more pregnancies vs. never pregnant; HR: 0.66, 95% CI: 0.46-0.96) in relapsing-onset patients, whereas no association was seen in progressive-onset patients after controlling for age at onset and immunomodulatory treatment. This study also showed that a later age at menarche

(⩾13) was associated with a lower hazard (HR: 0.61, 95% CI: 0.44-0.85) of disability progression in progressive-onset patients, but no association was seen in relapsing-onset patients. Thus, the internal consistency of this study was poor. With the measurement of pregnancies after MS onset, another retrospective cohort study[157] with 445 women also found that parous women had a decreased hazard of reaching EDSS 4 and 6 (HR: 0.55, 95% CI: 0.36-0.86, p=0.008, and HR: 0.42, 95% CI: 0.22-0.82, p=0.012, respectively). Ramagopalan and colleagues[158] found that the association between having children after MS onset and decreased hazard of reaching EDSS 6 could be fully accounted for by age of MS onset. Another hospital-based retrospective cohort study[159] with 277 women with MS found that the number of children was not associated with the hazard of progression to SPMS (OR: 0.93, 95% CI: 0.50-1.72, p=0.81). Including 254 pregnant and 423 non-pregnant women, Karp and colleagues[160] found that pregnancy did not have a long-term effect on MS clinical course. Several limitations in these studies limited the ability to make conclusions from these results, including retrospective design[156 157 159], non-population-based study populations[137138159], and the possibility of reverse causality between pregnancy and disability progression[138] (i.e., those with greater disability may have less children). Therefore, prospective cohort studies are needed to identify the association between preceding parity and subsequent disease activity and disability progression.

2.5.4 Stressful life events

Psychological comorbidity such as depression and anxiety is common in patients with MS[161]. A hypothesis about psychological stress and MS onset was proposed in the 19th century[162]. Evidence about the association of SLEs and MS development is still sparse and controversial[163-169]. Some large cohort studies have tested the hypothesis

that SLEs in childhood and adulthood predispose to an increased risk of MS. Riise et al conducted a large prospective cohort study[165] with two cohorts of female nurses (n = 121700; n = 116671), finding that general stressors at home or work during adulthood were not associated with the onset of MS (HR: 0.85, 95% CI: 0.32-2.26). This study also found that severe stressors such as physical and sexual abuse in childhood were also not related with MS development. With the follow-up time of approximately 63 million person-years in 3 million cases, another population-based cohort study[166]found that negative events prior to age 18 years (parental divorce, parental death, or death of a sibling) were associated with a higher risk of MS (RR: 1.10, 95% CI: 1.03-1.20). However, this significant association was solely driven by parental divorce (RR: 1.13, 95% CI: 1.04-1.23), whereas parental death or death of a sibling showed no association with MS development. A nationwide population-based cohort study[167]in Denmark found a higher hazard of MS onset (HR: 1.56; 95% CI: 1.05-2.31) in patients who lost a child unexpectedly. Higher exposure to adverse events in childhood was also found to be associated with an earlier onset in patients with MS (r=-0.3, p=0.04)[170]. However, the aforementioned studies did not control for possible confounders such as tobacco smoking or vitamin D levels, which may limit the validity of the results. Due to the inconsistent results and design deficits, no firm conclusions can be made from these studies. Current research does not support a major role of SLEs in MS development.

Mounting evidence has suggested that SLEs, especially negative SLEs, may play a role in MS exacerbations. Most patients believed that stressors were responsible for their exacerbations as well[171]. A meta-analysis[172] in 2003 including 14 studies demonstrated that previous SLEs were associated with a higher risk of subsequent

exacerbation ($p<0.0001$). A subsequent systematic review[173] found that most research showed predisposed SLEs were associated with a higher risk of MS onset or exacerbations. Due to the diversity of SLE measurement, MS diagnosis, study design, and patients recruited, meta-analysis was not suitable to perform and no firm conclusion could be drawn from the included studies.

The link between MS and altered regulation of the hypothalamic-pituitary-adrenal (HPA) axis[174] supports the hypothesis that the psychobiological stress system interacts with the pathological process in MS natural history. Elevated glucocorticoid responsivity[175] in patients with MS also supports this pathological process. Research has also shown that impairment of the HPA axis is associated with disability progression[176] and brain atrophy[177] in MS patients.

Based on the findings that negative SLEs may induce MS exacerbations, some trials have explored the effects of psychiatric interventions in MS clinical course. Mohr and colleagues performed a randomised trial[178] including 121 relapsing-onset patients, finding that a stress management programme could reduce the MRI activity during the treatment period (24 weeks), whereas in the post-intervention period (24 weeks), the effects were not detectable. This study also found no difference in the clinical exacerbation or disability progression between the treatment group and the control group. Failure to maintain the behavioural interventions after the treatment session[179] may limit the internal validity of this study, but the association between behavioural intervention and MS clinical course is still uncertain.

2.5.5 Genetic factors

MS is caused by an interplay of environmental and genetic factors. Data on familial recurrence rates of multiple sclerosis support the aetiological roles of risk genes. A population-based study[11] in Sweden with 28,396 MS patients demonstrated the existence of parent-of-origin effect, however, the significantly increased relative risk was only observed among paternal relations but not maternal relations. The authors assumed that the "carter effect" may lead to this phenomenon, which means that male patients need more risk genes to develop disease. Collecting three cohorts of patients with MS in Australia, O'Gorman et al[12] found that the age-adjusted relative risk for siblings was 2.13, which was lower than that reported in the UK[13] and Canada[14]. Due to a similar overall genetic susceptibility between Australia and the northern hemisphere, this study suggests that environmental factors may explain the difference. All these studies demonstrated the phenomenon of familial aggregation in patients with MS.

The association between genes encoding the major histocompatibility complex (MHC) and susceptibility of MS has been discussed extensively. With the genome-wide association study (GWAS) approach, 6 loci in human leucocyte antigen (HLA) and 110 established variants outside the HLA have been confirmed to be associated with the onset of MS[127 184-186]. Among all suspected genetic variants, *HLA-DRB1*15:01* showed the single strongest association with MS onset[187] in both relapsing-onset and progressive-onset[186] patients.

A few studies[188 189] have evaluated the relationship between specific genetic risk alleles and MS clinical characteristics. A cross-sectional study[190] with 180 MS

patients in Japan found that the presence of *HLA-DRB1*04:05* genotype was associated with increased IgG index and oligoclonal IgG bands in CSF, whereas no association was seen between *HLA-DRB15* genotype and CSF findings. Using a GWAS approach, another large cross-sectional study[189] with 5142 MS patients also found that *HLA-DRB1*04* alleles were associated with the presence of oligoclonal bands in CSF. *HLA-DRB1*15* alleles in this study were associated with the status of oligoclonal bands.

With respect to the analysis of genetic factors in ASO, mounting studies showed that presence of *HLA-DRB1*1501* risk allele was associated with earlier onset[127 191 192]. With 154 patients available for genetic data, Bove et al[193] showed that patients with at least one *HLA-DRB1*1501* risk allele showed a 3.4-years earlier onset (95% CI: 26.42-20.37, p=0.028). Other genotypes such as *HLA-DRB1*0801*[194] also showed some effects on delaying the onset of first symptom for approximately 5 years (p=0.01). With the calculation of a genetic risk score of 107 established genetic variants, a subsequent study[195] found a significant interaction (p=0.014) between genetic risk score and phenotypes of MS on age at onset: a higher genetic risk score was associated with an earlier onset in relapsing-onset patients (r=-0.1, p=0.005) and a delayed onset in progressive-onset patients (r=0.07, p=0.15).

However, the association between genetic variation and MS clinical course is still uncertain[196]. When adding 61 established MS-associated SNPs to a MS genetic burden score, a cross-sectional study[197] with 1997 MS patients found that a higher score was associated with an earlier age at onset ($p=2.65 \times 10^{-4}$). However, this study found no relationship between the MS genetic burden score and MSSS. A prospective

cohort study[198] following 141 RRMS patients for an average of 2.3 years found that five MS risk-associated SNPs were associated with relapses, whereas no SNPs were associated with annualised change in disability. The study had limited power, and the associations were non-significant after adjusting for multiple comparisons. A subsequent study[40] with 127 participants with a diagnosis of a first demyelinating event at study entry found that seven non-HLA SNPs were associated with the hazards of conversion to MS and of relapses, and another seven non-HLA SNPs were associated with annualised change in disability as measured by EDSS. For this study, small sample size may limit the significance, as all associations became non-significant after multiple comparisons; however, dose dependency, internal validity, and positive results of cumulative genetic risk score assessment supported a potential relationship between genetic risk factors and MS disability progression.

2.6 Diagnosis of MS

Although in most cases, clinical symptoms and MRI signs are sufficient for the diagnosis of MS, differentiating it from other neurological demyelination diseases such as neuromyelitis optic spectrum disorder and acute disseminated encephalomyelitis is still difficult in atypical MS patients. Accuracy has been considerably increased due to advances in MRI and other examinations such as serological and genetic testing, but misdiagnosis still occurs. Since most therapies are only effective in patients in the relapsing-remitting phase, early diagnosis of MS is essential.

The first episode of demyelinating dysfunction with no evidence suggesting previous symptoms is referred to as CIS[1]. The definition of CIS is an episode of neurological

dysfunction (acute or subacute) that lasts more than 24 hours, without evidence of fever, infection, or encephalopathy. Spontaneous remission after the attack is the most essential clinical feature, whereas other signs such as onset at younger age and maximum deficit occurring at approximately 4 weeks after onset can further support the diagnosis[199]. The most common clinical symptoms for people with CIS are brain stem dysfunctions, partial myelitis, and unilateral optic neuritis[200]. Even with long-term follow-up, approximately one third of patients show a monophasic course and never convert to MS[70 201]. Slowly progressive symptoms that last over a period of months may suggest the diagnosis of primary progressive MS, and clinical signs such as asymmetric paraparesis and hemiparesis are comparatively more common in this sub-type[202].

Due to its high sensitivity, reproducibility, and non-invasiveness, MRI has been commonly implemented as a diagnostic tool. Standardised protocols (e.g., MAGNIMS) aid in the diagnosis of MS[203]. Abnormal MRIs are present in nearly all MS patients[204] and more than 80% of CIS patients[70]. Typical multifocal T2-weighted signs are mostly located at periventricular, juxtacortical, and infratentorial regions[205]. T1-weighted lesions mostly appear hypointense while being enhanced after the administration of gadolinium. Following 121 CIS patients for more than 5 years, Sombekkeet al[206] found the odds of conversion to MS (OR 3.53, 95% CI 1.52-8.17) was significantly higher when compared patients with focal lesions in spinal cord to patients who did not have. Recruitment of participants was from one tertiary referral centre may influence the generalisation of the results.

For patients with typical clinical symptoms and findings on MRI, the CSFtest is not necessary, but it still could provide supportive evidence for the diagnosis of MS. More than 90% of MS patients and 68.6% CIS patients[207]show elevated IgG oligoclonal bands in the CSF that are absent in serum[208]. Abnormal CSF results could also be found in some other inflammatory diseases of the CSF; thus, the chance of a false-positive result should be considered. Evoked potential is another method for a supported MS diagnosis, and it could identify the clinically silent lesions in the CNS[209] and dissemination of lesions in space. A test of aquaporin 4 IgG in serum can assist in the differentiation between MS and neuromyelitis optica spectrum disorder, which is now confirmed to be a different disease[210].

2.6.1 McDonald Criteria

Dissemination of lesions in space and time are essential for an MS diagnosis. Alternative diagnoses should be considered and excluded. In the past, the diagnosis of MS[211] was based on clinical grounds alone: two separate attacks from two or more lesions. However, with the developments in MRI, new criteria are not based on the clinical findings alone.

For the diagnosis of RRMS, dissemination in space requires one or more lesions in at least two of four locations: periventricular, juxtacortical, infratentorial, and spinal cord. Symptomatic lesions at the brain stem and spinal cord are not included. Dissemination in time from MRI scans could be presented as new lesions that emerge on follow-up scans or the simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions. It was shown that the diagnosis of MS could be made approximately 17 months earlier when the updated diagnostic criteria were

applied[212]; and, with the implementation of MRI, one third of patients can be diagnosed with MS during the first relapse.

Due to slow disease progression, the diagnosis of PPMS can be confirmed only after 1 year from disease onset. To satisfy the diagnostic standard of dissemination in space, two of the three requirements should be met: one or more T2-weighted lesion at three characteristic regions (periventricular, juxtacortical, and infratentorial), two or more lesions at the spinal cord, and positive findings in CSF.

Fulfilment of MRI criteria can also be found in patients with other CNS conditions, such as other inflammatory diseases, small vessel cerebrovascular disease, and even in healthy individuals[213 214]. Therefore, the application of MRI criteria is only suitable for patients with clinical symptoms suggestive of MS[199].

The previous discussion was based on the 2010 version of McDonald criteria. To facilitate an earlier diagnosis, some changes have been made in the 2017 version[1]. In summary: 1) for patients with a typical CIS, a dissemination in space (supported by clinical or MRI demonstration) and the positive test of CSF-specific oligoclonal bands allows the diagnosis of MS[2 3]; 2) for patients with supratentorial and infratentorial lesions or spinal cord syndrome, symptomatic lesions could be used to demonstrate dissemination in space or time[4 5], and; 3) cortical lesions can be used to demonstrate dissemination in space[6].

The diagnostic criteria has been evolved over the years. Some researchers have argued that the improvement in the sensitivity of diagnostic criteria may influence the risk of conversion. Recently, a prospective cohort study[7] (published in JAMA

neurology) following 229 patients for 65 months showed that the updated diagnostic criteria (2017) had an increased sensitivity (68% vs. 36%, $p<0.001$) and a decreased specificity (61% vs. 85%, $p<0.001$) compared to criteria 2010. Since no patient had a neuropathological confirmation of MS, using sensitivity and specify to evaluate the diagnostic criteria is somewhat problematic. Another cohort study following 131 patients presenting with symptoms suspicious for MS for 2 years found that 64% of the patients fulfilling McDonald 2010 criteria did not convert to CDMS[225]. Some limitations such as the registry-based design, insufficient time of follow-up, and higher rate of missing cases during follow-up may bias the results. However, some other studies[4 5] have suggested that inclusion of symptomatic lesions would not influence the specificity of the new diagnostic criteria. Following 1,107 CIS patients prospectively, Tintore et al[5] found that risk of second relapse was similar between patients with single asymptomatic lesion on MRI and patients with a symptomatic lesion. Following 30 patients' conversion from CIS to CDMS, another small study[4] with 30 patients found that the inclusion of symptomatic lesion led to the increase of sensitivity (80% to 87%) but the specificity remained stable (77% to 80%). In summary, the updated could obviously diagnose more MS patients with minor symptoms, however, some evidence still suggested that clinicians should bear in mind that the specificity may decrease when the new diagnostic criteria is applied, especially when early DMTs were administered.

2.7 Treatment of MS

2.7.1 Treatment for relapsing-remitting MS

Therapeutic options for RRMS have expanded markedly since the first drug (interferon-beta) was approved on 1993[221]. More than 10 disease modifying drugs

have been approved for the modulation of disease activity in MS patients at the relapsing stage. Although the growing number of RCTs has confirmed that disease modifying therapy can influence the accumulation of irreversible nerve damage, their long-term effects are still suboptimal. People with MS respond differently to the same treatment; therefore, personalised therapy is necessary. Unsatisfactory response calls for a prompt shift of the treatment, but side effects of the treatment should be evaluated carefully. Escalation therapy should be considered in patients who do not respond, whereas in the case of adverse effects, another lower risk drug should be tried.

Previous research focused primarily on the reduction of relapse frequency and disability accumulation. However, with the emergence of increasingly effective therapeutic options, researchers have shifted the goals of therapy towards the concept of "no evidence of disease activity"[222 223], complete prevention of relapses, MRI activity, and disability accumulation. The treatment for RRMS patients should be administered at an early stage to facilitate better control of the aberrant immune system[224 225]. A prospective cohort study[226] following 215 patients for a minimum of 7 years found that sustaining no disease activity for 2 years was associated with a better prognosis, as 78.3% did not have disability progression (EDSS change <0.5) after 7 years. However, 46% of patients could maintain no evidence of disease activity status for 1 year; however, the proportion decreased significantly to 27.5% during the second year and only 7.9% after 7 years. This study indicated that sustaining long-term no evidence of disease activity was a difficult target to achieve in clinical practice, although it could predict a reduced disability accrual. To monitor the treatment effects closely, serial MRI scans are critical. For patients with no response

to their current treatment, MRI scans should be acquired every 12 months or more frequently[227].

Following we will discuss some therapeutic options for RRMS, and several key studies are listed in Table 3. As a naturally occurring cytokine, interferon-β was the first drug approved for RRMS. The major effect of interferon-β is anti-inflammation[228]. A number of RCTs have supported that interferon-β could reduce the relapse rate and disability progression for patients diagnosed with RRMS[229-232]. Via the induction of the proliferation of glatiramer-acetate-specific lymphocytes, which can cross the blood-brain barrier, glatiramer acetate also has anti-inflammatory effects. Like interferon-β, however, glatiramer acetate does not delay the time to sustained disability progression[233].

As a sphingosine 1-phosphate (S1P) receptor modulator *in vivo*, fingolimod could limit autoreactive lymphocytes in lymph nodes to inhibit their infiltration into the CNS. Fingolimod was ineffective in the MS animal model (experimental autoimmune encephalomyelitis, EAE) that was selectively deficient for S1P1 on astrocytes, which suggested that fingolimod had direct effects in the CNS. A 24-month RCT trial[234] with 1033 RRMS patients found that the annualised relapse rate decreased from 0.4 to 0.18 when compared with the placebo group. Patients treated with fingolimod were also superior to the placebo group with regard to other endpoints such as disability progression and disease activity on MRI.

Teriflunomide[235] inhibits the proliferation of autoreactive B and T lymphocytes and induces the proliferation of anti-inflammation cytokine profiles. Three RCTs[235-237]

have been conducted and have found that the efficacy of teriflunomide was similar to that of interferon-β.

Ocrelizumab is a monoclonal antibody that could inhibit CD20+ B cells. Conducting an RCT with 1656 RRMS patients[238], ocrelizumab significantly reduced the relapse rate by approximately 50%, and compared with interferon-β, it reduced the disability progression by 40%.

2.7.2 Treatment for progressive MS

Compared with treatment for RRMS, therapies for PPMS are limited. As shown in Table 3, although two studies[239 240] have shown a beneficial effect of interferon-β on PPMS patients, other studies all showed negative results[241-244]. A subsequent combined analysis[245] found that the divergent results of interferon-β on progressive MS patients resulted from the degree of disease activity of the chosen samples, and that samples enriched with patients with high relapse rates and MRI lesion activity were associated with positive findings. As discussed previously, fingolimod could directly enter the CNS, so it was hypothesised that it may slow the disability progression in patients with PPMS. However, a recent RCT[246] study found that there was no effect of fingolimod administration on disability progression. All these studies suggested that anti-inflammatory therapy could not slow disability progression in patients with PPMS. Based on the theory that B cells play an essential role in the pathogenesis of PPMS, one RCT study[247] with 439 PPMS patients found that the volume of T2 lesions significantly decreased ($p<0.001$), although the progression of confirmed disability was not slowed. However, a subgroup analysis restricted to patients aged less than 51 years showed a significant association between rituximab

use and delayed time to confirmed disability progression (HR: 0.52, p=0.010). According to these results, another phase 3 RCT study[248] recruiting participants with relatively younger age and shorter disease duration found that 12-week/24-week confirmed disability progression was delayed in patients with ocrelizumab use when compared to a placebo group (HR: 0.76, 95% CI: 0.59-0.98, p=0.03; HR: 0.75, 95% CI: 0.58-0.98, p=0.04). Secondary endpoints such as volume of T2 lesions and total brain volume loss were also significantly different between the ocrelizumab group and the placebo group. Disparity in the results between two anti-CD20+ B cell monoclonal antibodies may result from the demographic differences among the chosen samples[249].

Table 2.3 Key studies assessing association between treatment and MS clinical course

First Author (year)	research design	Sample size	main findings
PRISMS (1998)[231]	RCT, test association between interferon-β and disease activity in RRMS patients	dose 22 μg n = 189 dose 44 μg n = 184 Placebo n = 187	Time to confirmed disability progression delayed (p<0.05)
Kappos (2015)[250]	RCT, test association between daclizumab and disease activity in RRMS patients	daclizumab n = 919 interferon-β n = 922	Daclizumab vs. interferon β ARR (0.22 vs. 0.39, p<0.001) New or enlarged T2 lesions (4.2 vs. 9.4, p<0.001) confirmed disability progression (16% vs. 20%, p=0.16)
Kappos (2010)[234]	RCT, test association between fingolimod and disease activity in RRMS patients	dose 1.25 mg n = 429 dose 0.50 mg n = 425 Placebo n = 418	Fingolimod 1.25 mg ARR: 0.16 (0.13-0.19); EDSS: –0.03 ±0.88 Fingolimod 0.5 mg ARR: 0.18 (0.15-0.22); EDSS: 0.00 ±0.88 Placebo ARR: 0.40 (0.34-0.47); EDSS: 0.13 ±0.94
European Study Group (1998)[239]	RCT, test association between interferon-β and disability in SPMS patients	Interferon-β n = 360 Placebo n = 358	Time to confirmed disability progression: (OR: 0.65, 95% CI: 0.52-0.83)
Cohen (2002)[240]	RCT, test association between interferon-β and disability in SPMS patients	Interferon-β n = 217 Placebo n = 219	MSFC Z-score change was reduced 40.4% in IFNβ-1a subjects (p=0.033)
SPECTRIMS (2001)[241]	RCT, test association between interferon-β and disability in SPMS	dose 22 μg n = 209 dose 44 μg n =	Time to confirmed disability progression: (interferon-β 44 mg vs. placebo: HR: 0.83, 95% CI: 0.65-1.07;

	patients	204 Placebo n = 205	p=0.146)
Panitch (2004)[242]	RCT, test association between interferon-β and disability in SPMS patients	250 μg/m^2 n = 209 160 μg/m^2 n = 314 Placebo n = 308	Time to confirmed disability progression: (250 μg vs. placebo; p=0.61)
Andersen (2004)[243]	RCT, test association between interferon-β and disability in SPMS patients	Interferon-β n = 186 Placebo n = 178	Time to confirmed disability progression: (HR: 1.13, 95% CI: 0.82-1.57; p=0.45)
Wolinsky (2007)[244]	RCT, test association between Glatiramer and disability in PPMS patients	Glatiramer n = 627 Placebo n = 316	Time to confirmed disability progression: (HR: 0.87, 95% CI: 0.71-1.07; p=0.18)
Lublin (2016)[246]	RCT, test association between fingolimod and disability in PPMS patients	Fingolimod n = 336 Placebo n = 487	Time to confirmed disability progression: (HR: 0.95, 95% CI: 0.80-1.12; p=0.54)
Hawker (2009)[247]	RCT, test association between rituximab and disability in PPMS patients	Rituximab n = 336 Placebo n = 487	Time to confirmed disease progression: (38.5% placebo, 30.2% rituximab; p=0.14)
Montalban (2016)[248]	RCT, test association between ocrelizumab and disability in PPMS patients	Ocrelizumab n = 488 Placebo n = 244	Time to confirmed disability progression: (HR: 0.76, 95% CI: 0.59-0.98; p=0.03)

RCT, randomised controlled trial; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; HR, hazard ratio; CI, confidence interval; OR, odds ratio; EDSS, expanded disability status scale; ARR, annualised relapse rate; MSFC, MS functional composite.

2.7.3 Complementary and alternative treatments

In conjunction with pharmacological treatments, complementary and alternative treatments (CAMs) such as cannabis, diet, exercise, and psychological approaches are commonly applied to ease the symptoms in patients with MS. During the clinical course, almost every patient with MS reports having tried CAMs[251].

Some RCT studies showed that cannabis extract such as Sativex or tetrahydrocannabinol was associated with improvements in incontinence[252], pain[253], muscle stiffness,[254] and spasticity[255 256] in patients with MS. While the literature is still limited, current studies favour the beneficial effects of cannabis administration. The effects of vitamin D on patients with MS have been discussed previously. Here we

will discuss another dietary extract: polyunsaturated fatty acid (PUFA). Torkildsen et al[257] conducted a RCT study with 92 patients with MS, and they found that PUFA supplementation was not associated with relapse rate or disability progression. They also found no difference in fatigue and quality of life between the PUFA supplementation group and the control group. With more than 80% follow-up, this study was classified as class-II RCT according to Evidence-Based Spine- Care Journal (2013)'s standard[258]. Another RCT study found that additional supplementation of PUFA was associated with decreased relapse rate and disability level, however, small sample size (n=65), great loss of follow-up (35%) and not using a rigorous statistical method made it difficult to interpreter the results[259].Current studies could not conclude the association between diet and MS clinical course, however, converging evidence did not support a beneficial effect of dietary supplementation (vitamin D or PUFA) in patients with MS.

A number of studies showed that exercise could improve the ability of mobility[260] such as walking speed[261-263], muscle endurance/strength[264 265], and balance[262 265 266] in patients with MS. A meta-analysis[267] with 600 MS patients found that exercise training improved walking mobility among MS patients with the effect size of 0.19 (95% CI: 0.09-0.28). Some studies also showed a significant association between exercise and the improvement of psychological status[262 263]. However, an association between exercise and MS clinical course is still less certain, although some studies showed some evidence of the association between physical activity and atrophy of brain volume[268] and relapse[269]. Short duration of follow-up[270] and absence of a correct temporal relationship[271] still hinder the reliability of current evidence.

A number of RCT studies showed that cognitive-behavioural therapy (CBT), which is the most common studied psychological CAM, could improve the quality of life[272], anxiety[273], depression[273 274], fatigue[272 274-276], and distress[277] in patients with MS. However, like exercise studies, the current literature does not provide a robust evidence supporting the beneficial effects of psychological intervention on MS clinical course. As previous discussed, the RCT study conducted by Mohr et al[178] showed the reduction of enhancing lesions ($p=0.04$) in patients with stress management therapy could last only 24 weeks post-treatment.

Overall, the current treatments can partly prevent the occurrence of disease activity, whereas for disability progression and conversion to progressive disease, none of the treatments were effective. Effects of CAMs should be considered rigorously based on current studies. Therefore, the prevention of MS progression from other perspectives such as the modification of environmental/behavioural factors is needed. Also, the use of lifestyle interventions that could complement existing treatment is appealing for patients, as they generally have no side effects and result in an increased level of perceived control over the disease.

2.8 Summary

MS is a demyelinating disease of the CNS, and according to the pattern of relapses and disability progression, it can be divided into relapsing-onset disease and progressive-onset disease. Inflammation is responsible for the disease activity, whereas disability progression is largely due to neurodegeneration. Environmental risk factors such as smoking, low vitamin D levels, low exposure to UVR, and high immune response to EBV infection increased the risk of MS at the population level.

However, their role in MS disease activity and disability progression is less certain. Translating the findings to intervention and prevention of MS is still immature. Dissemination of lesions in time and space is the core to the diagnostic criteria of MS. Current treatments can partly prevent disease activity for patients with RRMS, and a phase 3 trial supported the effects of ocrelizumab on patients with PPMS.

2.9 Postscript

This chapter provided some key information, which will aid in the understanding of the rest of the thesis. However, this chapter has not discussed the association between viral infections and MS development and progression, which will be discussed in the next chapter.

2.10 References

1. Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* 2014;83(3):278-86. doi: 10.1212/wnl.0000000000000560 [published Online First: 2014/05/30]
2. Tremlett H, Zhao Y, Joseph J, et al. Relapses in multiple sclerosis are age- and time-dependent. *Journal of neurology, neurosurgery, and psychiatry*2008;79(12):1368-74. doi: 10.1136/jnnp.2008.145805 [published Online First: 2008/06/07]
3. Scalfari A, Neuhaus A, Degenhardt A, et al. The natural history of multiple sclerosis, a geographically based study 10: relapses and long-term disability. *Brain : a journal of neurology* 2010;133(7):1914-29. doi: 10.1093/brain/awq118
4. Lassmann H. Multiple Sclerosis Pathology. *Cold Spring Harbor perspectives in medicine* 2018;8(3) doi: 10.1101/cshperspect.a028936 [published Online First: 2018/01/24]
5. Kutzelnigg A, Lucchinetti C, Stadelmann C, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain : a journal of neurology* 2005;128(Pt 11):2705-12.
6. Haider L, Zrzavy T, Hametner S, et al. The topograpy of demyelination and neurodegeneration in the multiple sclerosis brain. *Brain : a journal of neurology* 2016;139(Pt 3):807-15.
7. Lucchinetti CF, Popescu BF, Bunyan RF, et al. Inflammatory cortical demyelination in early multiple sclerosis. *The New England journal of medicine* 2011;365(23):2188-97. doi: 10.1056/NEJMoa1100648
8. Banwell B, Krupp L, Kennedy J, et al. Clinical features and viral serologies in

children with multiple sclerosis: a multinational observational study. *Lancet neurology* 2007;6(9):773-81.
9. Alotaibi S, Kennedy J, Tellier R, et al. Epstein-Barr virus in pediatric multiple sclerosis. *JAMA : the journal of the American Medical Association* 2004;291(15):1875-9. doi: 10.1001/jama.291.15.1875 [published Online First: 2004/04/22]
10. Pohl D, Krone B, Rostasy K, et al. High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. *Neurology* 2006;67(11):2063-5.
11. Boiko A, Gusev E, Sudomoina M, et al. Association and linkage of juvenile MS with HLA-DR2(15) in Russians. *Neurology* 2002;58(4):658-60.
12. Renoux C, Vukusic S, Confavreux C. The natural history of multiple sclerosis with childhood onset. *Clinical neurology and neurosurgery* 2008;110(9):897-904. doi: 10.1016/j.clineuro.2008.04.009
13. Mowry E, Pesic M, Grimes B, et al. Demyelinating events in early multiple sclerosis have inherent severity and recovery. *Neurology* 2009;72(7):602-8.
14. O'Mahony J, Marrie R, Laporte A, et al. Recovery From Central Nervous System Acute Demyelination in Children. *Pediatrics* 2015;136(1):e115-23.
15. Harding K, Liang K, Cossburn M, et al. Long-term outcome of paediatric-onset multiple sclerosis: a population-based study. *J Neurol Neurosurg Psychiatry* 2013;84(2):141-7.
16. Bove R, Chitnis T. The role of gender and sex hormones in determining the onset and outcome of multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458513519181 [published Online First: 2014/02/25]
17. Waldman A, Ness J, Pohl D, et al. Pediatric multiple sclerosis: Clinical features and outcome. *Neurology* 2016;87(9 Suppl 2):S74-81.
18. Banwell B, Reder A, Krupp L, et al. Safety and tolerability of interferon beta-1b in pediatric multiple sclerosis. *Neurology* 2006;66(4):472-6.
19. Alroughani R, Boyko A. Pediatric multiple sclerosis: a review. *BMC neurology*2018;18(1):27. doi: 10.1186/s12883-018-1026-3 [published Online First: 2018/03/11]
20. Chitnis T, Tenembaum S, Banwell B, et al. Consensus statement: evaluation of new and existing therapeutics for pediatric multiple sclerosis. *Mult Scler* 2012;18(1):116-27.
21. Noseworthy J, Paty D, Wonnacott T, et al. Multiple sclerosis after age 50. *Neurology* 1983;33(12):1537-44. [published Online First: 1983/12/01]
22. Bove R, Healy B, Augustine A, et al. Effect of gender on late-onset multiple sclerosis. *Mult Scler* 2012;18(10):1472-9.
23. Martinelli V, Rodegher M, Moiola L, et al. Late onset multiple sclerosis: clinical characteristics, prognostic factors and differential diagnosis. *Neurol Sci* 2004;25 Suppl 4:S350-5.
24. Kis B, Rumberg B, Berlit P. Clinical characteristics of patients with late-onset multiple sclerosis. *J Neurol* 2008;255(5):697-702.
25. Scalfari A, Neuhaus A, Daumer M, et al. Age and disability accumulation in multiple sclerosis. *Neurology* 2011;77(13):1246-52.
26. Alroughani R, Akhtar S, Ahmed S, et al. Is Time to Reach EDSS 6.0 Faster in Patients with Late-Onset versus Young-Onset Multiple Sclerosis? *PloS one* 2016;11(11):e0165846. doi: 10.1371/journal.pone.0165846 [published Online

First: 2016/11/02]
27. Guillemin F, Baumann C, Epstein J, et al. Older Age at Multiple Sclerosis Onset Is an Independent Factor of Poor Prognosis: A Population-Based Cohort Study. *Neuroepidemiology* 2017;48(3-4):179-87. doi: 10.1159/000479516 [published Online First: 2017/08/10]
28. Cierny D, Lehotsky J, Hanysova S, et al. The age at onset in Multiple Sclerosis is associated with patient's prognosis. *Bratislavske lekarske listy* 2017;118(6):374-77. doi: 10.4149/bll_2017_071 [published Online First: 2017/07/01]
29. Confavreux C, Vukusic S. Natural history of multiple sclerosis: a unifying concept. *Brain : a journal of neurology* 2006;129(Pt 3):606-16. doi: 10.1093/brain/awl007
30. Ruet A, Deloire MS, Ouallet JC, et al. Predictive factors for multiple sclerosis in patients with clinically isolated spinal cord syndrome. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2011;17(3):312-8. doi: 10.1177/1352458510386999 [published Online First: 2010/11/13]
31. Kuhle J, Disanto G, Dobson R, et al. Conversion from clinically isolated syndrome to multiple sclerosis: A large multicentre study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015;21(8):1013-24. doi: 10.1177/1352458514568827 [published Online First: 2015/02/15]
32. Scalfari A, Lederer C, Daumer M, et al. The relationship of age with the clinical phenotype in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(13):1750-58. doi: 10.1177/1352458516630396
33. Tintore M, Rovira A, Rio J, et al. Defining high, medium and low impact prognostic factors for developing multiple sclerosis. *Brain : a journal of neurology* 2015;138(Pt 7):1863-74. doi: 10.1093/brain/awv105
34. Spelman T, Meyniel C, Rojas JI, et al. Quantifying risk of early relapse in patients with first demyelinating events: Prediction in clinical practice. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016:1352458516679893. doi: 10.1177/1352458516679893 [published Online First: 2016/11/26]
35. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Annals of neurology* 2011;69(2):292-302. doi: 10.1002/ana.22366
36. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Annals of neurology* 2005;58(6):840-6. doi: 10.1002/ana.20703 [published Online First: 2005/11/12]
37. Kurtzke JF. A new scale for evaluating disability in multiple sclerosis. *Neurology* 1955;5(8):580-3. [published Online First: 1955/08/01]
38. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 1983;33(11):1444-52. [published Online First: 1983/11/01]
39. Wang Y, Meyerson L, Tang Y, et al. Statistical methods for the analysis of relapse data in MS clinical trials. *J Neurol Sci* 2009;285(1-2):206-11.
40. Pan G, Simpson S, Jr., van der Mei I, et al. Role of genetic susceptibility variants in predicting clinical course in multiple sclerosis: a cohort study. *Journal of neurology, neurosurgery, and psychiatry* 2016;87(11):1204-11. doi: 10.1136/jnnp-2016-313722 [published Online First: 2016/08/26]

41. Simpson S, Jr., Taylor B, Dwyer DE, et al. Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(6):799-806. doi: 10.1177/1352458511428081 [published Online First: 2011/11/16]
42. Gaetani L, Fanelli F, Riccucci I, et al. High risk of early conversion to multiple sclerosis in clinically isolated syndromes with dissemination in space at baseline. *Journal of the neurological sciences* 2017;379:236-40. doi: 10.1016/j.jns.2017.06.008 [published Online First: 2017/07/19]
43. Giorgio A, Battaglini M, Rocca MA, et al. Location of brain lesions predicts conversion of clinically isolated syndromes to multiple sclerosis. *Neurology* 2013;80(3):234-41. doi: 10.1212/WNL.0b013e31827debeb [published Online First: 2012/12/12]
44. Ruet A, Arrambide G, Brochet B, et al. Early predictors of multiple sclerosis after a typical clinically isolated syndrome. *Multiple Sclerosis Journal* 2014;20(13):1721-26. doi: 10.1177/1352458514533397
45. Kalincik T, Vaneckova M, Tyblova M, et al. Volumetric MRI markers and predictors of disease activity in early multiple sclerosis: a longitudinal cohort study. *PloS one* 2012;7(11):e50101. doi: 10.1371/journal.pone.0050101
46. Repovic P, Lublin FD. Treatment of multiple sclerosis exacerbations. *Neurologic clinics* 2011;29(2):389-400. doi: 10.1016/j.ncl.2010.12.012
47. Uitdehaag BM, Barkhof F, Coyle PK, et al. The changing face of multiple sclerosis clinical trial populations. *Current medical research and opinion* 2011;27(8):1529-37. doi: 10.1185/03007995.2011.591370
48. Duddy M, Lee M, Pearson O, et al. The UK patient experience of relapse in Multiple Sclerosis treated with first disease modifying therapies. *Multiple sclerosis and related disorders* 2014;3(4):450-6. doi: 10.1016/j.msard.2014.02.006
49. Harding K, Tilling K, MacIver C, et al. Seasonal variation in multiple sclerosis relapse. *Journal of neurology* 2017;264(6):1059-67. doi: 10.1007/s00415-017-8485-0 [published Online First: 2017/04/21]
50. Kalincik T, Buzzard K, Jokubaitis V, et al. Risk of relapse phenotype recurrence in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514528762 [published Online First: 2014/04/30]
51. Bermel RA, Weinstock-Guttman B, Bourdette D, et al. Intramuscular interferon beta-1a therapy in patients with relapsing-remitting multiple sclerosis: a 15-year follow-up study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(5):588-96. doi: 10.1177/1352458509360549 [published Online First: 2010/02/20]
52. Bermel RA, You X, Foulds P, et al. Predictors of long-term outcome in multiple sclerosis patients treated with interferon beta. *Annals of neurology* 2013;73(1):95-103. doi: 10.1002/ana.23758
53. Ford C, Goodman AD, Johnson K, et al. Continuous long-term immunomodulatory therapy in relapsing multiple sclerosis: results from the 15-year analysis of the US prospective open-label study of glatiramer acetate. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(3):342-50. doi: 10.1177/1352458509358088 [published Online First: 2010/01/29]
54. Goodin DS, Jones J, Li D, et al. Establishing long-term efficacy in chronic disease: use of recursive partitioning and propensity score adjustment to

estimate outcome in MS. *PloS one* 2011;6(11):e22444. doi: 10.1371/journal.pone.0022444 [published Online First: 2011/12/06]
55. Goodin DS, Reder AT, Ebers GC, et al. Survival in MS: a randomized cohort study 21 years after the start of the pivotal IFNbeta-1b trial. *Neurology* 2012;78(17):1315-22. doi: 10.1212/WNL.0b013e3182535cf6 [published Online First: 2012/04/13]
56. Goodin DS, Traboulsee A, Knappertz V, et al. Relationship between early clinical characteristics and long term disability outcomes: 16 year cohort study (follow-up) of the pivotal interferon beta-1b trial in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2012;83(3):282-7. doi: 10.1136/jnnp-2011-301178 [published Online First: 2011/12/24]
57. Goodin DS, Reder AT, Bermel RA, et al. Relapses in multiple sclerosis: Relationship to disability. *Multiple sclerosis and related disorders* 2016;6:10-20. doi: 10.1016/j.msard.2015.09.002 [published Online First: 2016/04/12]
58. Scalfari A, Neuhaus A, Degenhardt A, et al. The natural history of multiple sclerosis: a geographically based study 10: relapses and long-term disability. *Brain : a journal of neurology* 2010;133(Pt 7):1914-29. doi: 10.1093/brain/awq118
59. Leray E, Yaouanq J, Le Page E, et al. Evidence for a two-stage disability progression in multiple sclerosis. *Brain : a journal of neurology* 2010;133(Pt 7):1900-13. doi: 10.1093/brain/awq076 [published Online First: 2010/04/29]
60. Compston A. Making progress on the natural history of multiple sclerosis. *Brain : a journal of neurology* 2006;129(Pt 3):561-3. doi: 10.1093/brain/awl034
61. Dunn SE, Gunde E, Lee H. Sex-Based Differences in Multiple Sclerosis (MS): Part II: Rising Incidence of Multiple Sclerosis in Women and the Vulnerability of Men to Progression of this Disease. *Current topics in behavioral neurosciences* 2015;26:57-86. doi: 10.1007/7854_2015_370 [published Online First: 2015/02/19]
62. Kalincik T, Vivek V, Jokubaitis V, et al. Sex as a determinant of relapse incidence and progressive course of multiple sclerosis. *Brain* 2013;136(Pt 12):3609-17. doi: 10.1093/brain/awt281
63. Sormani MP, Bonzano L, Roccatagliata L, et al. Magnetic resonance imaging as a potential surrogate for relapses in multiple sclerosis: a meta-analytic approach. *Annals of neurology* 2009;65(3):268-75. doi: 10.1002/ana.21606 [published Online First: 2009/04/01]
64. Sormani MP, Bruzzi P. MRI lesions as a surrogate for relapses in multiple sclerosis: a meta-analysis of randomised trials. *Lancet neurology* 2013;12(7):669-76. doi: 10.1016/s1474-4422(13)70103-0 [published Online First: 2013/06/08]
65. Fahrbach K, Huelin R, Martin AL, et al. Relating relapse and T2 lesion changes to disability progression in multiple sclerosis: a systematic literature review and regression analysis. *BMC neurology* 2013;13:180. doi: 10.1186/1471-2377-13-180 [published Online First: 2013/11/20]
66. Sormani MP, Bonzano L, Roccatagliata L, et al. Surrogate endpoints for EDSS worsening in multiple sclerosis. A meta-analytic approach. *Neurology* 2010;75(4):302-9. doi: 10.1212/WNL.0b013e3181ea15aa
67. Sormani MP, Bonzano L, Roccatagliata L, et al. Magnetic resonance imaging as surrogate for clinical endpoints in multiple sclerosis: data on novel oral drugs.

Multiple sclerosis (Houndmills, Basingstoke, England) 2011;17(5):630-3. doi: 10.1177/1352458510393770 [published Online First: 2010/12/24]
68. Dobson R, Rudick RA, Turner B, et al. Assessing treatment response to interferon-beta: is there a role for MRI? *Neurology* 2014;82(3):248-54. doi: 10.1212/wnl.0000000000000036 [published Online First: 2013/12/18]
69. Popescu V, Agosta F, Hulst HE, et al. Brain atrophy and lesion load predict long term disability in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2013;84(10):1082-91. doi: 10.1136/jnnp-2012-304094
70. Fisniku LK, Brex PA, Altmann DR, et al. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain : a journal of neurology* 2008;131(Pt 3):808-17. doi: 10.1093/brain/awm329 [published Online First: 2008/02/01]
71. Uher T, Horakova D, Bergsland N, et al. MRI correlates of disability progression in patients with CIS over 48 months. *NeuroImage Clinical* 2014;6:312-9. doi: 10.1016/j.nicl.2014.09.015 [published Online First: 2014/11/08]
72. Ruano L, Portaccio E, Goretti B, et al. Age and disability drive cognitive impairment in multiple sclerosis across disease subtypes. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2017;23(9):1258-67. doi: 10.1177/1352458516674367 [published Online First: 2016/10/16]
73. Lynch SG, Parmenter BA, Denney DR. The association between cognitive impairment and physical disability in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2005;11(4):469-76. doi: 10.1191/1352458505ms1182oa [published Online First: 2005/07/27]
74. Groman E, Bayer P, Kunze U, et al. [Analysis of the needs for diagnosis and therapy of tobacco dependence in Austria]. *Wien Med Wochenschr* 2000;150(6):109-14.
75. Marrie RA, Cutter G, Tyry T, et al. Smoking status over two years in patients with multiple sclerosis. *Neuroepidemiology* 2009;32(1):72-9. doi: 10.1159/000170910
76. Hawkes CH. Smoking is a risk factor for multiple sclerosis: a metanalysis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2007;13(5):610-5. doi: 10.1177/1352458506073501
77. Hernan MA, Olek MJ, Ascherio A. Cigarette smoking and incidence of multiple sclerosis. *American journal of epidemiology* 2001;154(1):69-74.
78. Riise T, Nortvedt MW, Ascherio A. Smoking is a risk factor for multiple sclerosis. *Neurology* 2003;61(8):1122-4. [published Online First: 2003/10/29]
79. Sundstrom P, Nystrom L, Hallmans G. Smoke exposure increases the risk for multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2008;15(6):579-83. doi: 10.1111/j.1468-1331.2008.02122.x [published Online First: 2008/05/14]
80. Degelman M, Herman K. Smoking and multiple sclerosis: A systematic review and meta-analysis using the Bradford Hill criteria for causation. *Multiple sclerosis and related disorders* 2017;17:207-16.
81. Moszczynski P, Zabinski Z, Moszczynski P, Jr., et al. Immunological findings in cigarette smokers. *Toxicol Lett* 2001;118(3):121-7.
82. Smith KJ, Kapoor R, Hall SM, et al. Electrically active axons degenerate when exposed to nitric oxide. *Annals of neurology* 2001;49(4):470-6. [published Online First: 2001/04/20]

83. Van Houten WH, Friede RL. Histochemical studies of experimental demyelination produced with cyanide. *Experimental neurology* 1961;4:402-12. [published Online First: 1961/11/01]
84. Gardner MJ, McCarthy TL, Jusko WJ. Relationship of serum thiocyanate concentrations to smoking characteristics. *Journal of toxicology and environmental health* 1984;14(2-3):393-406. doi: 10.1080/15287398409530588 [published Online First: 1984/01/01]
85. Correale J, Farez MF. Smoking worsens multiple sclerosis prognosis: two different pathways are involved. *Journal of neuroimmunology* 2015;281:23-34. doi: 10.1016/j.jneuroim.2015.03.006 [published Online First: 2015/04/14]
86. Wingerchuk DM. Smoking: effects on multiple sclerosis susceptibility and disease progression. *Therapeutic advances in neurological disorders* 2012;5(1):13-22. doi: 10.1177/1756285611425694
87. van der Mei I, Lucas RM, Taylor BV, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(4):461-9. doi: 10.1177/1352458515594040 [published Online First: 2015/07/23]
88. Mikaeloff Y, Caridade G, Tardieu M, et al. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. *Brain : a journal of neurology* 2007;130(Pt 10):2589-95. doi: 10.1093/brain/awm198 [published Online First: 2007/09/11]
89. Montgomery SM, Bahmanyar S, Hillert J, et al. Maternal smoking during pregnancy and multiple sclerosis amongst offspring. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2008;15(12):1395-9. doi: 10.1111/j.1468-1331.2008.02331.x
90. Hedstrom AK, Baarnhielm M, Olsson T, et al. Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2011;17(7):788-93. doi: 10.1177/1352458511399610 [published Online First: 2011/03/05]
91. Baltar VT, Xun WW, Chuang SC, et al. Smoking, secondhand smoke, and cotinine levels in a subset of EPIC cohort. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2011;20(5):869-75. doi: 10.1158/1055-9965.epi-10-1235 [published Online First: 2011/03/02]
92. Salzer J, Hallmans G, Nystrom M, et al. Smoking as a risk factor for multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(8):1022-7. doi: 10.1177/1352458512470862 [published Online First: 2012/12/22]
93. Di Pauli F, Reindl M, Ehling R, et al. Smoking is a risk factor for early conversion to clinically definite multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(8):1026-30. doi: 10.1177/1352458508093679 [published Online First: 2008/07/18]
94. Pittas F, Ponsonby AL, van der Mei IA, et al. Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis. *Journal of neurology* 2009;256(4):577-85. doi: 10.1007/s00415-009-0120-2
95. D'Hooghe M B, Haentjens P, Nagels G, et al. Alcohol, coffee, fish, smoking and

disease progression in multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2012;19(4):616-24. doi: 10.1111/j.1468-1331.2011.03596.x [published Online First: 2011/11/29]
96. Horakova D, Zivadinov R, Weinstock-Guttman B, et al. Environmental factors associated with disease progression after the first demyelinating event: results from the multi-center SET study. *PloS one* 2013;8(1):e53996. doi: 10.1371/journal.pone.0053996
97. Kappus N, Weinstock-Guttman B, Hagemeier J, et al. Cardiovascular risk factors are associated with increased lesion burden and brain atrophy in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2016;87(2):181-7. doi: 10.1136/jnnp-2014-310051 [published Online First: 2015/02/28]
98. Hernan MA, Jick SS, Logroscino G, et al. Cigarette smoking and the progression of multiple sclerosis. *Brain : a journal of neurology* 2005;128(Pt 6):1461-5. doi: 10.1093/brain/awh471
99. Healy BC, Ali EN, Guttmann CR, et al. Smoking and disease progression in multiple sclerosis. *Archives of neurology* 2009;66(7):858-64. doi: 10.1001/archneurol.2009.122
100. Ramanujam R, Hedstrom AK, Manouchehrinia A, et al. Effect of Smoking Cessation on Multiple Sclerosis Prognosis. *JAMA neurology* 2015;72(10):1117-23. doi: 10.1001/jamaneurol.2015.1788 [published Online First: 2015/09/09]
101. Koch M, van Harten A, Uyttenboogaart M, et al. Cigarette smoking and progression in multiple sclerosis. *Neurology* 2007;69(15):1515-20. doi: 10.1212/01.wnl.0000277658.78381.db
102. Munger KL, Fitzgerald KC, Freedman MS, et al. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. *Neurology* 2015;85(19):1694-701. doi: 10.1212/wnl.0000000000002099 [published Online First: 2015/10/11]
103. Kvistad S, Myhr KM, Holmoy T, et al. No association of tobacco use and disease activity in multiple sclerosis. *Neurology(R) neuroimmunology & neuroinflammation* 2016;3(4):e260. doi: 10.1212/nxi.0000000000000260 [published Online First: 2016/07/28]
104. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. *Neurology* 2008;71(2):129-35. doi: 10.1212/01.wnl.0000316802.35974.34
105. Taylor BV, Lucas RM, Dear K, et al. Latitudinal variation in incidence and type of first central nervous system demyelinating events. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(4):398-405. doi: 10.1177/1352458509359724 [published Online First: 2010/02/20]
106. Simpson S, Jr., Blizzard L, Otahal P, et al. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *Journal of neurology, neurosurgery, and psychiatry* 2011;82(10):1132-41. doi: 10.1136/jnnp.2011.240432
107. Disanto G, Pakpoor J, Morahan JM, et al. Epstein-Barr virus, latitude and multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(3):362-5. doi: 10.1177/1352458512451942
108. Willer CJ, Dyment DA, Sadovnick AD, et al. Timing of birth and risk of

multiple sclerosis: population based study. *Bmj* 2005;330(7483):120. doi: 10.1136/bmj.38301.686030.63
109. Handel AE, Giovannoni G, Ebers GC, et al. Environmental factors and their timing in adult-onset multiple sclerosis. *Nature reviews Neurology* 2010;6(3):156-66. doi: 10.1038/nrneurol.2010.1 [published Online First: 2010/02/17]
110. Sloka S, Silva C, Pryse-Phillips W, et al. A quantitative analysis of suspected environmental causes of MS. *The Canadian journal of neurological sciences Le journal canadien des sciences neurologiques* 2011;38(1):98-105.
111. van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *Bmj* 2003;327(7410):316. doi: 10.1136/bmj.327.7410.316
112. Bjornevik K, Riise T, Casetta I, et al. Sun exposure and multiple sclerosis risk in Norway and Italy: The EnvIMS study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(8):1042-9. doi: 10.1177/1352458513513968 [published Online First: 2014/01/15]
113. Holick MF. Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *The American journal of clinical nutrition* 2004;79(3):362-71. [published Online First: 2004/02/27]
114. Lucas RM, Ponsonby AL, Dear K, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011;76(6):540-8. doi: 10.1212/WNL.0b013e31820af93d
115. Kampman MT, Wilsgaard T, Mellgren SI. Outdoor activities and diet in childhood and adolescence relate to MS risk above the Arctic Circle. *Journal of neurology* 2007;254(4):471-7. doi: 10.1007/s00415-006-0395-5 [published Online First: 2007/03/23]
116. Staples J, Ponsonby AL, Lim L. Low maternal exposure to ultraviolet radiation in pregnancy, month of birth, and risk of multiple sclerosis in offspring: longitudinal analysis. *Bmj* 2010;340:c1640. doi: 10.1136/bmj.c1640
117. Wion D, MacGrogan D, Neveu I, et al. 1,25-Dihydroxyvitamin D3 is a potent inducer of nerve growth factor synthesis. *Journal of neuroscience research* 1991;28(1):110-4. doi: 10.1002/jnr.490280111 [published Online First: 1991/01/01]
118. Gogal RM, Jr., Holladay SD. Perinatal TCDD exposure and the adult onset of autoimmune disease. *J Immunotoxicol* 2008;5(4):413-8. doi: 10.1080/10408360802483201
119. Ascherio A, Munger KL, Giovannucci E. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011;77(14):1405; author reply 05-6. doi: 10.1212/WNL.0b013e3182294610
120. Becklund BR, Severson KS, Vang SV, et al. UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production. *Proceedings of the National Academy of Sciences of the United States of America* 2010;107(14):6418-23. doi: 10.1073/pnas.1001119107
121. Holick MF. Vitamin D deficiency. *The New England journal of medicine* 2007;357(3):266-81. doi: 10.1056/NEJMra070553 [published Online First: 2007/07/20]
122. Stroud ML, Stilgoe S, Stott VE, et al. Vitamin D - a review. *Australian family physician* 2008;37(12):1002-5. [published Online First: 2009/01/15]

123. Saltyte Benth J, Myhr KM, Loken-Amsrud KI, et al. Modelling and prediction of 25-hydroxyvitamin D levels in Norwegian relapsing-remitting multiple sclerosis patients. *Neuroepidemiology* 2012;39(2):84-93. doi: 10.1159/000339360 [published Online First: 2012/07/21]
124. van der Mei IA, Simpson S, Knippenberg S, et al. Role of vitamin D in multiple sclerosis: implications for disease management. *Neurodegenerative disease management* 2011;1(6):523-36.
125. Ramagopalan S, Dyment D, Cader M, et al. Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Ann Neurol* 2011;70(6):881-6.
126. Ramagopalan SV, Maugeri NJ, Handunnetthi L, et al. Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. *PLoS genetics* 2009;5(2):e1000369. doi: 10.1371/journal.pgen.1000369 [published Online First: 2009/02/07]
127. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011;476(7359):214-9. doi: 10.1038/nature10251
128. Zhuang JC, Huang ZY, Zhao GX, et al. Variants of CYP27B1 are associated with both multiple sclerosis and neuromyelitis optica patients in Han Chinese population. *Gene* 2015;557(2):236-9. doi: 10.1016/j.gene.2014.12.045 [published Online First: 2014/12/30]
129. Harroud A, Richards J. Mendelian randomization in multiple sclerosis: A causal role for vitamin D and obesity? *Mult Scler* 2018;24(1):80-85.
130. Mokry L, Ross S, Ahmad O, et al. Vitamin D and Risk of Multiple Sclerosis: A Mendelian Randomization Study. *PLoS Med* 2015;12(8):e1001866.
131. Rhead B, Baarnhielm M, Gianfrancesco M, et al. Mendelian randomization shows a causal effect of low vitamin D on multiple sclerosis risk. *Neurology Genetics* 2016;2(5):e97. doi: 10.1212/nxg.0000000000000097 [published Online First: 2016/09/22]
132. Munger KL, Levin LI, Hollis BW, et al. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA : the journal of the American Medical Association* 2006;296(23):2832-8. doi: 10.1001/jama.296.23.2832
133. Gelfand J, Cree B, McElroy J, et al. Vitamin D in African Americans with multiple sclerosis. *Neurology* 2011;76(21):1824-30.
134. Jin J. Vitamin D and Calcium Supplements for Preventing Fractures. *JAMA : the journal of the American Medical Association* 2018;319(15):1630.
135. Simpson S, Taylor B, Blizzard L, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS. *Annals of neurology* 2010:n/a-n/a. doi: 10.1002/ana.22043
136. Mowry EM, Waubant E, McCulloch CE, et al. Vitamin D status predicts new brain magnetic resonance imaging activity in multiple sclerosis. *Annals of neurology* 2012;72(2):234-40. doi: 10.1002/ana.23591 [published Online First: 2012/08/29]
137. Martinelli V, Dalla Costa G, Colombo B, et al. Vitamin D levels and risk of multiple sclerosis in patients with clinically isolated syndromes. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(2):147-55. doi: 10.1177/1352458513494959 [published Online First: 2013/07/10]
138. Stewart N, Simpson S, Jr., van der Mei I, et al. Interferon-beta and serum 25-

hydroxyvitamin D interact to modulate relapse risk in MS. *Neurology* 2012;79(3):254-60. doi: 10.1212/WNL.0b013e31825fded9 [published Online First: 2012/06/16]
139. Pierrot-Deseilligny C, Rivaud-Pechoux S, Clerson P, et al. Relationship between 25-OH-D serum level and relapse rate in multiple sclerosis patients before and after vitamin D supplementation. *Therapeutic advances in neurological disorders* 2012;5(4):187-98. doi: 10.1177/1756285612447090 [published Online First: 2012/07/12]
140. Runia TF, Hop WC, de Rijke YB, et al. Lower serum vitamin D levels are associated with a higher relapse risk in multiple sclerosis. *Neurology* 2012;79(3):261-6. doi: 10.1212/WNL.0b013e31825fdec7 [published Online First: 2012/06/16]
141. Ascherio A, Munger KL, White R, et al. Vitamin D as an early predictor of multiple sclerosis activity and progression. *JAMA neurology* 2014;71(3):306-14. doi: 10.1001/jamaneurol.2013.5993 [published Online First: 2014/01/22]
142. Soilu-Hanninen M, Aivo J, Lindstrom BM, et al. A randomised, double blind, placebo controlled trial with vitamin D3 as an add on treatment to interferon beta-1b in patients with multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2012;83(5):565-71. doi: 10.1136/jnnp-2011-301876 [published Online First: 2012/03/01]
143. James E, Dobson R, Kuhle J, et al. The effect of vitamin D-related interventions on multiple sclerosis relapses: a meta-analysis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(12):1571-9. doi: 10.1177/1352458513489756 [published Online First: 2013/05/24]
144. Laursen JH, Sondergaard HB, Sorensen PS, et al. Vitamin D supplementation reduces relapse rate in relapsing-remitting multiple sclerosis patients treated with natalizumab. *Multiple sclerosis and related disorders* 2016;10:169-73. doi: 10.1016/j.msard.2016.10.005 [published Online First: 2016/12/07]
145. Pozuelo-Moyano B, Benito-León J, Mitchell AJ, et al. A Systematic Review of Randomized, Double-Blind, Placebo-Controlled Trials Examining the Clinical Efficacy of Vitamin D in Multiple Sclerosis. *Neuroepidemiology* 2013;40(3):147-53. doi: 10.1159/000345122
146. Koch-Henriksen N, Sorensen PS. The changing demographic pattern of multiple sclerosis epidemiology. *Lancet neurology* 2010;9(5):520-32. doi: 10.1016/S1474-4422(10)70064-8
147. Kotzamani D, Panou T, Mastorodemos V, et al. Rising incidence of multiple sclerosis in females associated with urbanization. *Neurology* 2012;78(22):1728-35. doi: 10.1212/WNL.0b013e31825830a9 [published Online First: 2012/05/18]
148. Orton SM, Herrera BM, Yee IM, et al. Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet neurology* 2006;5(11):932-6. doi: 10.1016/s1474-4422(06)70581-6 [published Online First: 2006/10/21]
149. Confavreux C, Hutchinson M, Hours MM, et al. Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *The New England journal of medicine* 1998;339(5):285-91. doi: 10.1056/NEJM199807303390501
150. Vukusic S, Hutchinson M, Hours M, et al. Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain : a journal of*

neurology 2004;127(Pt 6):1353-60. doi: 10.1093/brain/awh152
151. Hughes SE, Spelman T, Gray OM, et al. Predictors and dynamics of postpartum relapses in women with multiple sclerosis. *Mult Scler* 2014;20(6):739-46. doi: 10.1177/1352458513507816
152. Finkelsztejn A, Brooks JB, Paschoal FM, Jr., et al. What can we really tell women with multiple sclerosis regarding pregnancy? A systematic review and meta-analysis of the literature. *BJOG* 2011;118(7):790-7. doi: 10.1111/j.1471-0528.2011.02931.x
153. Morales LB, Loo KK, Liu HB, et al. Treatment with an estrogen receptor alpha ligand is neuroprotective in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2006;26(25):6823-33. doi: 10.1523/JNEUROSCI.0453-06.2006
154. Nielsen NM, Jorgensen KT, Stenager E, et al. Reproductive history and risk of multiple sclerosis. *Epidemiology (Cambridge, Mass)* 2011;22(4):546-52. doi: 10.1097/EDE.0b013e31821c7adc
155. Ponsonby AL, Lucas RM, van der Mei IA, et al. Offspring number, pregnancy, and risk of a first clinical demyelinating event: the AusImmune Study. *Neurology* 2012;78(12):867-74. doi: 10.1212/WNL.0b013e31824c4648
156. D'Hooghe M B, Haentjens P, Nagels G, et al. Menarche, oral contraceptives, pregnancy and progression of disability in relapsing onset and progressive onset multiple sclerosis. *Journal of neurology* 2012;259(5):855-61. doi: 10.1007/s00415-011-6267-7 [published Online First: 2011/10/14]
157. Masera S, Cavalla P, Prosperini L, et al. Parity is associated with a longer time to reach irreversible disability milestones in women with multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015;21(10):1291-7. doi: 10.1177/1352458514561907
158. Ramagopalan S, Yee I, Byrnes J, et al. Term pregnancies and the clinical characteristics of multiple sclerosis: a population based study. *Journal of neurology, neurosurgery, and psychiatry* 2012;83(8):793-5. doi: 10.1136/jnnp-2012-302848
159. Koch M, Uyttenboogaart M, Heersema D, et al. Parity and secondary progression in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2009;80(6):676-8. doi: 10.1136/jnnp.2008.160911
160. Karp I, Manganas A, Sylvestre MP, et al. Does pregnancy alter the long-term course of multiple sclerosis? *Annals of epidemiology* 2014;24(7):504-8 e2. doi: 10.1016/j.annepidem.2014.04.007
161. Marrie RA, Reingold S, Cohen J, et al. The incidence and prevalence of psychiatric disorders in multiple sclerosis: a systematic review. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015;21(3):305-17. doi: 10.1177/1352458514564487
162. Charcot JM. Lectures on the diseases of the nervous system. London: New Sydenham Society, 1897.
163. Warren S, Greenhill S, Warren KG. Emotional stress and the development of multiple sclerosis: case-control evidence of a relationship. *Journal of chronic diseases* 1982;35(11):821-31.
164. Grant I, Brown GW, Harris T, et al. Severely threatening events and marked life difficulties preceding onset or exacerbation of multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 1989;52(1):8-13.

165. Riise T, Mohr DC, Munger KL, et al. Stress and the risk of multiple sclerosis. *Neurology* 2011;76(22):1866-71. doi: 10.1212/WNL.0b013e31821d74c5 [published Online First: 2011/06/01]
166. Nielsen NM, Pedersen BV, Stenager E, et al. Stressful life-events in childhood and risk of multiple sclerosis: a Danish nationwide cohort study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(12):1609-15. doi: 10.1177/1352458514528761 [published Online First: 2014/04/02]
167. Li J, Johansen C, Bronnum-Hansen H, et al. The risk of multiple sclerosis in bereaved parents: A nationwide cohort study in Denmark. *Neurology* 2004;62(5):726-9. [published Online First: 2004/03/10]
168. Spitzer C, Bouchain M, Winkler LY, et al. Childhood trauma in multiple sclerosis: a case-control study. *Psychosomatic medicine* 2012;74(3):312-8. doi: 10.1097/PSY.0b013e31824c2013 [published Online First: 2012/03/13]
169. Liu XJ, Ye HX, Li WP, et al. Relationship between psychosocial factors and onset of multiple sclerosis. *European neurology* 2009;62(3):130-6. doi: 10.1159/000226428 [published Online First: 2009/07/03]
170. Shaw MT, Pawlak NO, Frontario A, et al. Adverse Childhood Experiences Are Linked to Age of Onset and Reading Recognition in Multiple Sclerosis. *Frontiers in neurology* 2017;8:242. doi: 10.3389/fneur.2017.00242 [published Online First: 2017/06/20]
171. Simmons RD, Ponsonby AL, van der Mei IA, et al. What affects your MS? Responses to an anonymous, Internet-based epidemiological survey. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2004;10(2):202-11. doi: 10.1191/1352458504ms1006oa [published Online First: 2004/05/06]
172. Mohr DC, Hart SL, Julian L, et al. Association between stressful life events and exacerbation in multiple sclerosis: a meta-analysis. *Bmj* 2004;328(7442):731. doi: 10.1136/bmj.38041.724421.55
173. Artemiadis AK, Anagnostouli MC, Alexopoulos EC. Stress as a risk factor for multiple sclerosis onset or relapse: a systematic review. *Neuroepidemiology*2011;36(2):109-20. doi: 10.1159/000323953
174. Gold SM, Mohr DC, Huitinga I, et al. The role of stress-response systems for the pathogenesis and progression of MS. *Trends in immunology* 2005;26(12):644-52. doi: 10.1016/j.it.2005.09.010
175. Michelson D, Stone L, Galliven E, et al. Multiple sclerosis is associated with alterations in hypothalamic-pituitary-adrenal axis function. *The Journal of clinical endocrinology and metabolism* 1994;79(3):848-53. doi: 10.1210/jcem.79.3.8077372 [published Online First: 1994/09/01]
176. Gold SM, Raji A, Huitinga I, et al. Hypothalamo-pituitary-adrenal axis activity predicts disease progression in multiple sclerosis. *Journal of neuroimmunology* 2005;165(1-2):186-91. doi: 10.1016/j.jneuroim.2005.04.014 [published Online First: 2005/06/07]
177. Schumann EM, Kumpfel T, Then Bergh F, et al. Activity of the hypothalamic-pituitary-adrenal axis in multiple sclerosis: correlations with gadolinium-enhancing lesions and ventricular volume. *Annals of neurology* 2002;51(6):763-7. doi: 10.1002/ana.10187 [published Online First: 2002/07/12]
178. Mohr DC, Lovera J, Brown T, et al. A randomized trial of stress management for the prevention of new brain lesions in MS. *Neurology* 2012;79(5):412-9. doi:

10.1212/WNL.0b013e3182616ff9 [published Online First: 2012/07/13]
179. Nilsen WJ, Haverkos L, Nebeling L, et al. Maintenance of long-term behavior change. *Am J Health Behav* 2010;34(6):643-6.
180. Westerlind H, Ramanujam R, Uvehag D, et al. Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. *Brain : a journal of neurology* 2014;137(Pt 3):770-8.
181. O'Gorman C, Freeman S, Taylor B, et al. Familial recurrence risks for multiple sclerosis in Australia. *J Neurol Neurosurg Psychiatry* 2011;82(12):1351-4.
182. Robertson NP, Fraser M, Deans J, et al. Age-adjusted recurrence risks for relatives of patients with multiple sclerosis. *Brain : a journal of neurology* 1996;119 (Pt 2):449-55. [published Online First: 1996/04/01]
183. Ebers GC, Sadovnick AD, Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group. *Nature* 1995;377(6545):150-1. doi: 10.1038/377150a0
184. Moutsianas L, Jostins L, Beecham AH, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nature genetics* 2015;47(10):1107-13. doi: 10.1038/ng.3395
185. Baranzini SE, Wang J, Gibson RA, et al. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. *Human molecular genetics* 2009;18(4):767-78. doi: 10.1093/hmg/ddn388
186. Martinelli-Boneschi F, Esposito F, Brambilla P, et al. A genome-wide association study in progressive multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(10):1384-94. doi: 10.1177/1352458512439118
187. Ramagopalan SV, Knight JC, Ebers GC. Multiple sclerosis and the major histocompatibility complex. *Current opinion in neurology* 2009;22(3):219-25. doi: 10.1097/WCO.0b013e32832b5417
188. Buck D, Albrecht E, Aslam M, et al. Genetic variants in the immunoglobulin heavy chain locus are associated with the IgG index in multiple sclerosis. *Annals of neurology* 2013;73(1):86-94. doi: 10.1002/ana.23749 [published Online First: 2012/12/12]
189. Mero IL, Gustavsen MW, Saether HS, et al. Oligoclonal band status in Scandinavian multiple sclerosis patients is associated with specific genetic risk alleles. *PloS one* 2013;8(3):e58352. doi: 10.1371/journal.pone.0058352 [published Online First: 2013/03/09]
190. Niino M, Sato S, Fukazawa T, et al. Latitude and HLA-DRB1 alleles independently affect the emergence of cerebrospinal fluid IgG abnormality in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015 doi: 10.1177/1352458514560924
191. Masterman T, Ligers A, Olsson T, et al. HLA-DR15 is associated with lower age at onset in multiple sclerosis. *Annals of neurology* 2000;48(2):211-9.
192. Cree BA, Reich DE, Khan O, et al. Modification of Multiple Sclerosis Phenotypes by African Ancestry at HLA. *Archives of neurology* 2009;66(2):226-33. doi: 10.1001/archneurol.2008.541
193. Bove R, Chua AS, Xia Z, et al. Complex relation of HLA-DRB1*1501, age at menarche, and age at multiple sclerosis onset. *Neurology Genetics* 2016;2(4):e88. doi: 10.1212/nxg.0000000000000088 [published Online First: 2016/08/10]

194. Wu JS, Qiu W, Castley A, et al. Modifying effects of HLA-DRB1 allele interactions on age at onset of multiple sclerosis in Western Australia. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(1):15-20. doi: 10.1177/1352458509350312
195. Sorosina M, Esposito F, Guaschino C, et al. Inverse correlation of genetic risk score with age at onset in bout-onset and progressive-onset multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514561910
196. van der Walt A, Stankovich J, Bahlo M, et al. Apolipoprotein genotype does not influence MS severity, cognition, or brain atrophy. *Neurology* 2009;73(13):1018-25. doi: 10.1212/WNL.0b013e3181b9c85e [published Online First: 2009/09/30]
197. Harbo HF, Isobe N, Berg-Hansen P, et al. Oligoclonal bands and age at onset correlate with genetic risk score in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(6):660-8. doi: 10.1177/1352458513506503
198. Lin R, Taylor BV, Simpson S, Jr., et al. Association between multiple sclerosis risk-associated SNPs and relapse and disability--a prospective cohort study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(3):313-21. doi: 10.1177/1352458513496882 [published Online First: 2013/07/28]
199. Brownlee WJ, Hardy TA, Fazekas F, et al. Diagnosis of multiple sclerosis: progress and challenges. *Lancet* 2016 doi: 10.1016/s0140-6736(16)30959-x [published Online First: 2016/11/28]
200. Brownlee WJ, Miller DH. Clinically isolated syndromes and the relationship to multiple sclerosis. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 2014;21(12):2065-71. doi: 10.1016/j.jocn.2014.02.026
201. Swanton JK, Fernando K, Dalton CM, et al. Is the frequency of abnormalities on magnetic resonance imaging in isolated optic neuritis related to the prevalence of multiple sclerosis? A global comparison. *Journal of neurology, neurosurgery, and psychiatry* 2006;77(9):1070-2. doi: 10.1136/jnnp.2006.090910 [published Online First: 2006/06/22]
202. Miller DH, Leary SM. Primary-progressive multiple sclerosis. *Lancet neurology* 2007;6(10):903-12. doi: 10.1016/s1474-4422(07)70243-0 [published Online First: 2007/09/22]
203. Rovira A, Wattjes MP, Tintore M, et al. Evidence-based guidelines: MAGNIMS consensus guidelines on the use of MRI in multiple sclerosis-clinical implementation in the diagnostic process. *Nature reviews Neurology* 2015;11(8):471-82. doi: 10.1038/nrneurol.2015.106 [published Online First: 2015/07/08]
204. Offenbacher H, Fazekas F, Schmidt R, et al. Assessment of MRI criteria for a diagnosis of MS. *Neurology* 1993;43(5):905-9.
205. Fazekas F, Barkhof F, Filippi M, et al. The contribution of magnetic resonance imaging to the diagnosis of multiple sclerosis. *Neurology* 1999;53(3):448-56. [published Online First: 1999/08/17]
206. Sombekke MH, Wattjes MP, Balk LJ, et al. Spinal cord lesions in patients with clinically isolated syndrome: a powerful tool in diagnosis and prognosis. *Neurology* 2013;80(1):69-75. doi: 10.1212/WNL.0b013e31827b1a67

[published Online First: 2012/12/18]
207. Dobson R, Ramagopalan S, Davis A, et al. Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *Journal of neurology, neurosurgery, and psychiatry* 2013;84(8):909-14. doi: 10.1136/jnnp-2012-304695
208. Freedman MS, Thompson EJ, Deisenhammer F, et al. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. *Archives of neurology* 2005;62(6):865-70. doi: 10.1001/archneur.62.6.865 [published Online First: 2005/06/16]
209. Leocani L, Comi G. Clinical neurophysiology of multiple sclerosis. *Handbook of clinical neurology* 2014;122:671-9. doi: 10.1016/b978-0-444-52001-2.00028-5 [published Online First: 2014/02/11]
210. Wingerchuk DM, Banwell B, Bennett JL, et al. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. *Neurology* 2015;85(2):177-89. doi: 10.1212/WNL.0000000000001729
211. Poser CM, Paty DW, Scheinberg L, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Annals of neurology* 1983;13(3):227-31. doi: 10.1002/ana.410130302
212. Brownlee WJ, Swanton JK, Altmann DR, et al. Earlier and more frequent diagnosis of multiple sclerosis using the McDonald criteria. *Journal of neurology, neurosurgery, and psychiatry* 2015;86(5):584-5. doi: 10.1136/jnnp-2014-308675 [published Online First: 2014/11/22]
213. Liu S, Kullnat J, Bourdette D, et al. Prevalence of brain magnetic resonance imaging meeting Barkhof and McDonald criteria for dissemination in space among headache patients. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(8):1101-5. doi: 10.1177/1352458512471874 [published Online First: 2013/02/06]
214. Nielsen JM, Korteweg T, Barkhof F, et al. Overdiagnosis of multiple sclerosis and magnetic resonance imaging criteria. *Annals of neurology* 2005;58(5):781-3. doi: 10.1002/ana.20632 [published Online First: 2005/10/22]
215. Thompson A, Banwell B, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet neurology* 2018;17(2):162-73.
216. Huss A, Halbgebauer S, Öckl P, et al. Importance of cerebrospinal fluid analysis in the era of McDonald 2010 criteria: a German-Austrian retrospective multicenter study in patients with a clinically isolated syndrome. *J Neurol* 2016;263(12):2499-504.
217. Arrambide G, Tintore M, Espejo C, et al. The value of oligoclonal bands in the multiple sclerosis diagnostic criteria. *Brain : a journal of neurology* 2018;141(4):1075-84.
218. Brownlee W, Swanton J, Miszkiel K, et al. Should the symptomatic region be included in dissemination in space in MRI criteria for MS? *Neurology* 2016;87(7):680-3.
219. Tintore M, Otero-Romero S, R ó J, et al. Contribution of the symptomatic lesion in establishing MS diagnosis and prognosis. *Neurology* 2016;87(13):1368-74.
220. Filippi M, Preziosa P, Meani A, et al. Prediction of a multiple sclerosis diagnosis in patients with clinically isolated syndrome using the 2016 MAGNIMS and

2010 McDonald criteria: a retrospective study. *Lancet neurology* 2018;17(2):133-42.
221. Khan OA. Multiple sclerosis: a review of existing therapy and future prospects. *JPMA The Journal of the Pakistan Medical Association* 1996;46(1):20-4. [published Online First: 1996/01/01]
222. Imitola J, Racke MK. Is no evidence of disease activity a realistic goal for patients with multiple sclerosis? *JAMA neurology* 2015;72(2):145-7. doi: 10.1001/jamaneurol.2014.3860 [published Online First: 2014/12/23]
223. Bevan CJ, Cree BA. Disease activity free status: a new end point for a new era in multiple sclerosis clinical research? *JAMA neurology* 2014;71(3):269-70. doi: 10.1001/jamaneurol.2013.5486 [published Online First: 2014/01/08]
224. Trojano M, Pellegrini F, Paolicelli D, et al. Real-life impact of early interferon beta therapy in relapsing multiple sclerosis. *Annals of neurology* 2009;66(4):513-20. doi: 10.1002/ana.21757 [published Online First: 2009/10/23]
225. Khatri B, Barkhof F, Comi G, et al. Comparison of fingolimod with interferon beta-1a in relapsing-remitting multiple sclerosis: a randomised extension of the TRANSFORMS study. *Lancet neurology* 2011;10(6):520-9. doi: 10.1016/s1474-4422(11)70099-0 [published Online First: 2011/05/17]
226. Rotstein DL, Healy BC, Malik MT, et al. Evaluation of no evidence of disease activity in a 7-year longitudinal multiple sclerosis cohort. *JAMA neurology* 2015;72(2):152-8. doi: 10.1001/jamaneurol.2014.3537 [published Online First: 2014/12/23]
227. Rio J, Comabella M, Montalban X. Predicting responders to therapies for multiple sclerosis. *Nature reviews Neurology* 2009;5(10):553-60. doi: 10.1038/nrneurol.2009.139
228. Sellebjerg F, Datta P, Larsen J, et al. Gene expression analysis of interferon-beta treatment in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(5):615-21. doi: 10.1177/1352458507085976 [published Online First: 2008/04/15]
229. Paty DW, Li DK. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology* 1993;43(4):662-7. [published Online First: 1993/04/01]
230. Jacobs LD, Cookfair DL, Rudick RA, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Annals of neurology* 1996;39(3):285-94. doi: 10.1002/ana.410390304 [published Online First: 1996/03/01]
231. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. *Lancet* 1998;352(9139):1498-504. [published Online First: 1998/11/20]
232. Galea I, Ward-Abel N, Heesen C. Relapse in multiple sclerosis. *Bmj* 2015;350:h1765. doi: 10.1136/bmj.h1765
233. Johnson KP, Brooks BR, Ford CC, et al. Sustained clinical benefits of glatiramer

acetate in relapsing multiple sclerosis patients observed for 6 years. Copolymer 1 Multiple Sclerosis Study Group. *Mult Scler* 2000;6(4):255-66.
234. Kappos L, Radue EW, O'Connor P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *The New England journal of medicine* 2010;362(5):387-401. doi: 10.1056/NEJMoa0909494 [published Online First: 2010/01/22]
235. Claussen MC, Korn T. Immune mechanisms of new therapeutic strategies in MS: teriflunomide. *Clinical immunology* 2012;142(1):49-56. doi: 10.1016/j.clim.2011.02.011
236. O'Connor P, Wolinsky JS, Confavreux C, et al. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *The New England journal of medicine* 2011;365(14):1293-303. doi: 10.1056/NEJMoa1014656 [published Online First: 2011/10/14]
237. Confavreux C, O'Connor P, Comi G, et al. Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet neurology* 2014;13(3):247-56. doi: 10.1016/s1474-4422(13)70308-9 [published Online First: 2014/01/28]
238. Milo R. Therapeutic strategies targeting B-cells in multiple sclerosis. *Autoimmunity reviews* 2016;15(7):714-8. doi: 10.1016/j.autrev.2016.03.006 [published Online First: 2016/03/13]
239. Placebo-controlled multicentre randomised trial of interferon beta-1b in treatment of secondary progressive multiple sclerosis. European Study Group on interferon beta-1b in secondary progressive MS. *Lancet* 1998;352(9139):1491-7. [published Online First: 1998/11/20]
240. Cohen JA, Cutter GR, Fischer JS, et al. Benefit of interferon beta-1a on MSFC progression in secondary progressive MS. *Neurology* 2002;59(5):679-87. [published Online First: 2002/09/11]
241. Randomized controlled trial of interferon- beta-1a in secondary progressive MS: Clinical results. *Neurology* 2001;56(11):1496-504. [published Online First: 2001/06/13]
242. Panitch H, Miller A, Paty D, et al. Interferon beta-1b in secondary progressive MS: results from a 3-year controlled study. *Neurology* 2004;63(10):1788-95. [published Online First: 2004/11/24]
243. Andersen O, Elovaara I, Farkkila M, et al. Multicentre, randomised, double blind, placebo controlled, phase III study of weekly, low dose, subcutaneous interferon beta-1a in secondary progressive multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2004;75(5):706-10. [published Online First: 2004/04/20]
244. Wolinsky JS, Narayana PA, O'Connor P, et al. Glatiramer acetate in primary progressive multiple sclerosis: results of a multinational, multicenter, double-blind, placebo-controlled trial. *Annals of neurology* 2007;61(1):14-24. doi: 10.1002/ana.21079 [published Online First: 2007/01/31]
245. Kappos L, Weinshenker B, Pozzilli C, et al. Interferon beta-1b in secondary progressive MS: a combined analysis of the two trials. *Neurology* 2004;63(10):1779-87.
246. Lublin F, Miller DH, Freedman MS, et al. Oral fingolimod in primary progressive multiple sclerosis (INFORMS): a phase 3, randomised, double-blind, placebo-controlled trial. *Lancet* 2016;387(10023):1075-84. doi:

10.1016/S0140-6736(15)01314-8
247. Hawker K, O'Connor P, Freedman MS, et al. Rituximab in patients with primary progressive multiple sclerosis: results of a randomized double-blind placebo-controlled multicenter trial. *Annals of neurology* 2009;66(4):460-71. doi: 10.1002/ana.21867
248. Montalban X, Hauser SL, Kappos L, et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *The New England journal of medicine* 2016 doi: 10.1056/NEJMoa1606468 [published Online First: 2016/12/22]
249. Ontaneda D, Thompson AJ, Fox RJ, et al. Progressive multiple sclerosis: prospects for disease therapy, repair, and restoration of function. *Lancet* 2016 doi: 10.1016/s0140-6736(16)31320-4 [published Online First: 2016/11/28]
250. Kappos L, Wiendl H, Selmaj K, et al. Daclizumab HYP versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *The New England journal of medicine* 2015;373(15):1418-28. doi: 10.1056/NEJMoa1501481
251. Pucci E, Cartechini E, Taus C, et al. Why physicians need to look more closely at the use of complementary and alternative medicine by multiple sclerosis patients. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2004;11(4):263-7. doi: 10.1046/j.1468-1331.2003.00758.x [published Online First: 2004/04/06]
252. Freeman RM, Adekanmi O, Waterfield MR, et al. The effect of cannabis on urge incontinence in patients with multiple sclerosis: a multicentre, randomised placebo-controlled trial (CAMS-LUTS). *International urogynecology journal and pelvic floor dysfunction* 2006;17(6):636-41. doi: 10.1007/s00192-006-0086-x [published Online First: 2006/03/23]
253. Rog DJ, Nurmikko TJ, Friede T, et al. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology* 2005;65(6):812-9. doi: 10.1212/01.wnl.0000176753.45410.8b [published Online First: 2005/09/28]
254. Zajicek JP, Hobart JC, Slade A, et al. Multiple sclerosis and extract of cannabis: results of the MUSEC trial. *Journal of neurology, neurosurgery, and psychiatry* 2012;83(11):1125-32. doi: 10.1136/jnnp-2012-302468 [published Online First: 2012/07/14]
255. Collin C, Davies P, Mutiboko IK, et al. Randomized controlled trial of cannabis-based medicine in spasticity caused by multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2007;14(3):290-6. doi: 10.1111/j.1468-1331.2006.01639.x [published Online First: 2007/03/16]
256. Novotna A, Mares J, Ratcliffe S, et al. A randomized, double-blind, placebo-controlled, parallel-group, enriched-design study of nabiximols* (Sativex((R))), as add-on therapy, in subjects with refractory spasticity caused by multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2011;18(9):1122-31. doi: 10.1111/j.1468-1331.2010.03328.x [published Online First: 2011/03/03]
257. Torkildsen O, Wergeland S, Bakke S, et al. omega-3 fatty acid treatment in multiple sclerosis (OFAMS Study): a randomized, double-blind, placebo-controlled trial. *Archives of neurology* 2012;69(8):1044-51. doi: 10.1001/archneurol.2012.283 [published Online First: 2012/04/18]
258. Definition of the different classes of evidence (CoE). Evid Based Spine Care J

2013;4:167.
259. Rezapour-Firouzi S, Arefhosseini S, Mehdi F, et al. Immunomodulatory and therapeutic effects of Hot-nature diet and co-supplemented hemp seed, evening primrose oils intervention in multiple sclerosis patients. *Complementary therapies in medicine* 2013;21(5):473-80.
260. Collett J, Dawes H, Meaney A, et al. Exercise for multiple sclerosis: a single-blind randomized trial comparing three exercise intensities. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2011;17(5):594-603. doi: 10.1177/1352458510391836 [published Online First: 2011/01/21]
261. Romberg A, Virtanen A, Ruutiainen J, et al. Effects of a 6-month exercise program on patients with multiple sclerosis: a randomized study. *Neurology* 2004;63(11):2034-8.
262. Tarakci E, Yeldan I, Huseyinsinoglu BE, et al. Group exercise training for balance, functional status, spasticity, fatigue and quality of life in multiple sclerosis: a randomized controlled trial. *Clinical rehabilitation* 2013;27(9):813-22. doi: 10.1177/0269215513481047 [published Online First: 2013/04/02]
263. Garrett M, Hogan N, Larkin A, et al. Exercise in the community for people with minimal gait impairment due to MS: an assessor-blind randomized controlled trial. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(6):782-9. doi: 10.1177/1352458512461966 [published Online First: 2012/11/07]
264. Dodd KJ, Taylor NF, Shields N, et al. Progressive resistance training did not improve walking but can improve muscle performance, quality of life and fatigue in adults with multiple sclerosis: a randomized controlled trial. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2011;17(11):1362-74. doi: 10.1177/1352458511409084 [published Online First: 2011/06/17]
265. Straudi S, Fanciullacci C, Martinuzzi C, et al. The effects of robot-assisted gait training in progressive multiple sclerosis: A randomized controlled trial. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(3):373-84. doi: 10.1177/1352458515620933 [published Online First: 2015/12/15]
266. Gandolfi M, Munari D, Geroin C, et al. Sensory integration balance training in patients with multiple sclerosis: A randomized, controlled trial. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015;21(11):1453-62. doi: 10.1177/1352458514562438 [published Online First: 2015/01/15]
267. Snook EM, Motl RW. Effect of exercise training on walking mobility in multiple sclerosis: a meta-analysis. *Neurorehabilitation and neural repair* 2009;23(2):108-16. doi: 10.1177/1545968308320641 [published Online First: 2008/10/25]
268. Prakash RS, Snook EM, Motl RW, et al. Aerobic fitness is associated with gray matter volume and white matter integrity in multiple sclerosis. *Brain research* 2010;1341:41-51. doi: 10.1016/j.brainres.2009.06.063 [published Online First: 2009/06/30]
269. Pilutti LA, Platta ME, Motl RW, et al. The safety of exercise training in multiple sclerosis: a systematic review. *Journal of the neurological sciences* 2014;343(1-2):3-7. doi: 10.1016/j.jns.2014.05.016
270. Motl RW, McAuley E. Association between change in physical activity and short-term disability progression in multiple sclerosis. *Journal of rehabilitation medicine* 2011;43(4):305-10. doi: 10.2340/16501977-0782

[published Online First: 2011/02/10]
271. Tallner A, Waschbisch A, Wenny I, et al. Multiple sclerosis relapses are not associated with exercise. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(2):232-5. doi: 10.1177/1352458511415143 [published Online First: 2011/07/08]
272. Mohr DC, Hart SL, Julian L, et al. Telephone-administered psychotherapy for depression. *Archives of general psychiatry* 2005;62(9):1007-14. doi: 10.1001/archpsyc.62.9.1007
273. Lincoln NB, Yuill F, Holmes J, et al. Evaluation of an adjustment group for people with multiple sclerosis and low mood: a randomized controlled trial. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2011;17(10):1250-7. doi: 10.1177/1352458511408753 [published Online First: 2011/05/27]
274. Grossman P, Kappos L, Gensicke H, et al. MS quality of life, depression, and fatigue improve after mindfulness training: a randomized trial. *Neurology* 2010;75(13):1141-9. doi: 10.1212/WNL.0b013e3181f4d80d
275. van Kessel K, Moss-Morris R, Willoughby E, et al. A randomized controlled trial of cognitive behavior therapy for multiple sclerosis fatigue. *Psychosomatic medicine* 2008;70(2):205-13. doi: 10.1097/PSY.0b013e3181643065 [published Online First: 2008/02/08]
276. Thomas S, Thomas PW, Kersten P, et al. A pragmatic parallel arm multi-centre randomised controlled trial to assess the effectiveness and cost-effectiveness of a group-based fatigue management programme (FACETS) for people with multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2013;84(10):1092-9. doi: 10.1136/jnnp-2012-303816 [published Online First: 2013/05/23]
277. Moss-Morris R, Dennison L, Landau S, et al. A randomized controlled trial of cognitive behavioral therapy (CBT) for adjusting to multiple sclerosis (the saMS trial): does CBT work and for whom does it work? *J Consult Clin Psychol* 2013;81(2):251-62. doi: 10.1037/a0029132 [published Online First: 2012/06/27]

Chapter 3 Review of the association between human herpesvirus & human endogenous retrovirus and MS onset & progression

3.1 Preface

In this chapter, we will review the role of viral infections on MS development and progression, and some assumptions about the mechanisms underlying these associations. This chapter has been published in the *Journal of the Neurological Sciences*, (2017) vol 372, page 239-249. The typeset version of the manuscript as it appeared in the journal is in Appendix.

3.2 Abstract

This review discusses the role of EBV, HHV6 and human endogenous retroviruses (HERVs) in the onset and progression of MS. Although EBV has been established as one of the causal factors in MS onset, its role in MS progression is still uncertain. Moreover, interactions between EBV and other risk factor on MS development still need more investment. With less consistent evidence than EBV, HHV6 has also been implicated in the pathogenesis of MS; moreover, it showed a closer connection with the disease activity. Recent studies found that HERVs were associated with the development and progression of MS. Some antiviral treatments have shown promise for clinical interventions in the future. Future studies are yet needed to fully clarify the role of these agents in MS onset and disease course and the modes by which they realise these effects.

3.3 Introduction

Multiple sclerosis (MS) is a chronic and progressive inflammatory disease, causing demyelination, axonal loss and atrophy of the central nervous system. The aetiology of MS is due to interplay between genetic and environmental/behaviouralfactors[1 2]. For genetic factors, associations between the *HLA-DR* locus and MS have been consistently demonstrated, with the strongest of these being *HLA-DRB1*1501*[3 4]. As previously discussed in Chapter 2, a number of environmental and behavioural factors, including vitamin D/UV[5]and smoking[6],are now viewed as causal factors. In addition to these, viral agents, particularly Epstein-Barr virus (EBV), human herpesvirus 6 (HHV6)[7] and human endogenous retroviruses (HERVs)[8], are thought to have an involvement in MS onset and progression.

This review discusses the role of EBV, HHV6 and HERVs in the onset and progression of MS. It focuses on the key epidemiological studies investigating the role of these viruses in disease, as well as interactions between these viruses and other risk factors, and the potential mechanisms underlying these associations. Finally, the potential of interventions targeting these agents and their modes of effect in the prevention and treatment of MS will be discussed.

3.4 Epstein-Barr virus

3.4.1 What is Epstein-Barr virus?

EBV is a member of the human herpesvirus family and mainly infects B lymphocytes and epithelial cells. Viral parameters including Epstein-Barr nuclear antigen (EBNA) complex, which includes EBNA-1, 2, 3a, 3b, 3c and leader protein, viral capsid

antigen (VCA), early antigen (EA) and EBV DNA (used to define viral load) are often used in EBV research. EA is observed at the early phase of EBV infection and only expressed during the lytic stage (active replication). EBV DNA can be found in serum at initial infection or during reactivation from the latent phase. In a clinical and research setting, initial infection is characterised by the rise of IgM antibodies against VCA, while at and after convalescence, these anti-VCA IgM antibodies decrease while IgG against VCA and EBNA-1 emerge and increase [9]. EBNA-2 antibodies appear in the acute phase of EBV infection and decline during convalescence, whereas EBNA-1 antibodies become detectable during convalescence and remain stable[10].

Infected B-cells are immortalised and remain infected for life. Most infected B-cells are removed from circulation after the attack of cytotoxic T lymphocytes, while some remaining infected B-cells survive and enter the latent phase. EBV genomes exist as episomes residing in roughly one per million B-cells at this stage[11]. According to the variation of genetic expression in different cell lines, the latent stage is divided into three programs: type I is characterised by the multiplication of EBV genomes while B-cells are dividing, while B-cell differentiation and activation of naive B-cells are the feature of type II and type III, respectively. EBV genomes exist as episomes residing in one in a million B cells at this stage[11]. However, while other mechanism of reactivation may exist, subsequent infections of the EBV-infected B-cells may induce stimulation and activation of the B-cell from the latent phase (reviewed in reference[12]). In addition to the constant expression of EBNA-1 in all three latency stages, other EBNAs and LMPs are only expressed in the second and/or third latency stage. During

the latency stage, persistent EBV-infected B-cells are under control by cell-mediated immunity[13]; however, in conditions of lowered immunocompetency, EBV infection is associated with higher risk of malignancies[14].

EBV infection during childhood is usually asymptomatic, infection at or after adolescence manifests as a symptomatic infection termed infectious mononucleosis (IM) or glandular fever.

3.4.2 Prevalence of EBV infection

The marked difference in seropositivity between MS patients and healthy controls was one of the earliest findings implicating EBV as a risk factor in MS. While EBV is ubiquitous in the general population (~90%), in MS patients this approaches 100%[15 16]. This being so, EBV infection is thought to be a necessary component of MS aetiology, and it has recently been suggested that EBV would be detected in every MS patient if detection techniques were sufficiently sensitive[17]. A meta-analysis reported that when two different detection techniques were used, no clinically definite MS patients were EBV negative[15].

3.4.3 Infectious mononucleosis and MS risk

It has been consistently demonstrated that a history of IM has a positive relationship with the subsequent occurrence of MS[18], with meta-analyses[19 20] demonstrating a relative risk for MS greater than 2.0. The effect of IM on subsequent MS risk is long-lasting[21]. A direct relationship between latitude and variation in winter ambient UV has led to the hypothesis[22 23] that differences in winter ambient UV underlies the oft-cited latitudinal gradient in MS prevalence and incidence[24 25]. However, others have

suggested that different frequencies of exposure to EBV - particularly infections after childhood leading to IM[18 26] - may also contribute to this variability by latitude. Both IM and MS exhibit a positive latitudinal gradient of prevalence in temperate latitudes but are rare at tropical latitudes, and have a positive association with socioeconomic status[27]. However, no consideration of some important confounders such as ethnicity, socioeconomic status, and environmental/behavioural factors may have influenced the validity of the findings. Certainly, however, EBV, UV and other factors are not mutually exclusive, and may even interact with one another[28 29].

3.4.4 EBV biomarkers and MS onset

The association between a history of infection and the risk of MS onset has been evaluated by many case-control studies and pooled analyses[15 30 31], all which showed a strong relationship between EBV seropositivity and MS risk. Also supportive of a true association is the very strong and dose-dependent associations observed for anti-EBV serological parameters, with the strongest associations seen for anti-EBNA (anti-EBNA complex, anti-EBNA 1 and anti-EBNA2 all showed significant association with MS risk) [32-35]. Moreover, the evidence supporting the temporal relationship has been shown in studies where EBV exposure parameters were measured prior to MS onset[32-36].

Direct detection of EBV DNA, referred to as viral load, in cerebrospinal fluid (CSF) or peripheral blood suggests a role for EBV reactivation in MS clinical course. In the absence of a molecular mimicry mode of action (discussed later), such reactivation is the most obvious direct mechanism by which the virus could modulate disease risk and particularly disease course later in life. However, the frequency of EBV DNA

positivity in CSF or peripheral blood has generally shown no clear differences between MS patients and controls[37-40]. Assessing EBV DNA in both latent and lytic phases through examining the viral load from peripheral blood mononuclear cells (PBMCs) and serum also found a no difference when comparing 55 MS patients with 51 matched other neurological disease patients[37]. Actually, EBV DNA has been infrequently detected in any body fluid at any stage of MS[41]. Detection of antibodies to EA also indicates EBV reactivation, but as for EBV viral load, most studies could not find an association between anti-EA Ig and MS risk: a recent meta-analysis including 14 studies showed no association[31]. These results suggest there is no strong link between EBV reactivation and MS risk.

3.4.5 EBV biomarkers and MS clinical course

The links found between EBV infection and MS risk led naturally to investigations for a role for EBV in MS clinical course, including relapse, disability progression and magnetic resonance imaging (MRI)-detected disease activity. Most studies, although not all[42], have found that EBV reactivation (anti-EA IgG or EBV DNA) is infrequent during the progression of MS, and not associated with disease activity[41 43-45]. Following 19 MS patients for 1 year, IgA, IgM and serum DNA to EBV were found only in patients during exacerbations[42]. Although the study design was a prospective cohort study, the 19 patients were selected from 108 hospital-based CDMS patients caused the selection bias and decreased the external validity of the results. Another study found that levels of EBV-specific $CD8^+$ T-cells were more common during active disease compared to remission (19% vs. 39%, $p=0.05$)[46], suggesting that the ability to control EBV infection was associated with disease activity. Perhaps

supporting this finding, a single SPMS case study wherein supplementation with autologous anti-EBV $CD8^+$ T-cells produced measurable clinical benefits[47].

Anti-EBNA IgG is the parameter most frequently used to analyse immune response to EBV and its association with MS disease activity. Current studies have suggested that anti-EBNA is stable during MS course[34], one study even finding that anti-EBNA remained stable after the use of disease modifying therapy[48]. In a prospective study of 147 clinically isolated syndrome patients, elevated anti-EBNA1-IgG titres were associated with higher hazard of conversion to MS (McDonald Criteria[49]) during seven years of follow-up (HR=2.2, 95% CI:1.2-4.3; p=0.003)[50], but adjustment for other covariates attenuated the association and rendered it nonsignificant (HR=1.6, 95% CI: 0.9-3.0; p=0.13).A recent prospective cohort study[51] also found no association between baseline anti-EBNA1 levels and subsequent conversion to CDMS, and our study[52] also found no association with relapse in prevalent cases. A paediatric study found a stronger effect between EBV seropositivity and subsequent CDMS risk in 247 patients (HR 3.58, 95% CI 1.62-7.91)[53], leaving the possibility open for a true association.

Two longitudinal studies[45 50] with 5 and 7 years of follow-up, respectively, showed a positive association between anti-EBNA1 IgG titres and increase in EDSS. Anti-EBNA1 IgG titres were significantly correlated with change in EDSS (r^2=0.3, p=0.004), while another study found baseline anti-EBNA1 IgG titres were associated with EDSS in the first and fifth year of follow-up, respectively (r=0.21; p=0.015 & r=0.25; p=0.01). Associations between anti-EBV immune response with subclinical MRI-detected pathological changes, including lesion metrics and atrophy, were

another method to evaluate the relationship with clinical course. For instance, lesion volume or number measured by MRI has been associated with anti-EBNA1 IgG titre[45] [50] [54]. Anti-VCA IgG was also found to be associated with MRI-detected total brain atrophy and increased lesion volume[55].

Overall, the association between EBV biomarkers and MS activity is less consistent than that seen for onset. Evidence tends to support a relationship between anti-EBV level and MRI activity or disability progression, but is less convincing for relapse or conversion to CDMS. If a significant relationship between EBV infection and MS clinical course exists, the impact on MS pathology may not be substantial enough to alter clinical symptoms as the observation interval of most studies was less than five years. Longer observation periods are required to better understand the relationship, if any, with clinical course.

3.4.6 Association between EBV and other risk factors on MS risk

Beyond direct associations of EBV infection and clinical outcomes, EBV has been shown to interact with other risk factors in predicting MS onset.

EBV & genetic risk factors

A number of studies[56-61] (Table) demonstrated a significant interaction between EBV infection and *HLA-DR15* on MS risk. A significant additive interaction between history of IM and *HLA-DR15* genotype (Synergy index, 3.78; p=0.03), indicating that the combined effect of IM and*HLA-DR15* was larger than expected based on the sum of their individual effects, was found using a multicentre case-control study[56] with

282 cases with a first clinical diagnosis of demyelination. Another case-control study[57] also found a significant additive interaction, with synergy index 2.09. Multiplicative interaction between IM and *HLA-DR15* was not significant in most studies, however[56 57 62]. In terms of levels of immune response to EBV, most studies[56 62-65] have found no significant interaction between anti-EBNA IgG and *HLA-DR15*. One genome-wide association study[66] (n=1,956) in Mexican-American population found that genetic variation in the HLA region (nearest gene: *HLA-DRB1,* SNP rs2516049) was associated with the levels of anti-EBNA1-IgG ($p=1.4x10^{-15}$), and this association was specific to anti-EBNA as no association was observed for 12 other pathogens. A subsequent study[67] in an Australian twin cohort (n=3,599) further supported this significant association (p=4.11x10-9), moreover, a positive relationship between 3 of the 110 non-HLA SNPs associated with MS risk and anti-EBNA1-IgG was found in this study when combined in a meta-analysis with the previous dataset. The genes found included EVI5, which is an important mediator of viral incorporation and EOMES, again associated with host response to viral infections. These genes have a biologically plausible association with host responses to viral infections and are associated with increased MS risk.

EBV & vitamin D/sun exposure

Of great interest is the potential interaction of EBV parameters with other major environmental risk factors, such as vitamin D and sun exposure. The most direct association between EBV infection and vitamin D on MS risk is that EBNA3 can bind to the vitamin D receptor (VDR)[68] and may attenuate vitamin D-related immunoregulatory functions[69]. Therefore, the higher immune response to EBV may

hinder the protective role of vitamin D on MS onset. However, given the established association between EBNA1 and MS onset, epidemiological research has focused mostly on this component. Also, EBNA3 comprises a small proportion of total EBNA, with EBNA1 comprising the majority. No significant correlation was found between $25(OH)D_3$ and EBV load (r=0.10, p=0.33) or anti-EBNA1 IgG (r=0.11, p=0.43) in a cross-sectional study with 196 healthy participants.[70]A recent randomised-control study[71] with 68 RRMS patients found that levels of antibody targeting a region of EBNA-1 (amino acids 385-420) significantly decreased as a result of vitamin D supplementation (20,000 IU/week) after 48 weeks, although the effect was reduced and non-significant after 96 weeks.

Our case-control study[56] demonstrated that neither additive and multiplicative interaction between low 25(OH)D and EBV infection (history of IM & anti-EBNA level) reached significance. In support of this finding, another case-control study[72] also showed no interaction between low 25(OH)D and higher anti-EBNA.

EBV & smoking

The epidemiological evidence in support of an interaction between EBV and smoking is conflicting. As in Table, a pooled analysis of 442 patients and 865 controls found a significant interaction with the association between EBNA1 and MS stronger among ever smokers compared to never smokers (test for interaction $p<0.001$)[64]. Three other case-control studies failed to show any significant interaction[56 61 73]. Design of studies, sources of the cases, and timing at which samples were collected may be responsible

for the mixed and conflicting findings, but current evidence does not support a significant interaction between EBV infection and tobacco smoking on MS onset.

Table 3.1 Key studies for the interaction between EBV infection & other risk factors on MS onset

Author	Study information	Interactions assessed	Main findings
van der Mei[56]& colleagues (2015)[a]	Case-control, 282 FCD & 558 controls	EBV infection & other risk factors	1.Additive interaction: IM &*HLA-DR15* was significant (SI 3.78, p=0.03) IM & Low 25(OH)D was not significant (p=0.54) IM & smoking was not significant (p=0.62) anti-EBNA IgG &*HLA-DR15* was not significant (p=0.41) anti-EBNA IgG Low 25(OH)D & was not significant (p=0.69) anti-EBNA IgG & smoking was not significant (p=0.63) 2.Multiplicative interaction: IM &*HLA-DR15* was not significant (p=0.15) IM & Low 25(OH)D was not significant (p=0.25) IM & smoking was not significant (p=0.97) anti-EBNA IgG &*HLA-DR15* was not significant (p=0.95) anti-EBNA IgG & Low 25(OH)D was not significant (p=0.37) anti-EBNA IgG & smoking was not significant (p=0.46)
Disanto[57]& colleagues (2013)[b]	Case-control, 733 MS & 1089 controls	IM & DR15	1.Multiplicative interaction between *HLA-DR15*& IM was not significant (p=0.27) 2.Additive interaction between *HLA-DR15*& IM was significant (SI: 2.09, 95% CI: 1.59-2.59)
Sundqvist[59]& colleagues (2012)[c]	Case-control, 512 MS & 672 controls	EBV infection & HLA	1. Additive interaction between *HLA-DR15*& IM was significant (AP 0.34, 95%CI 0.01-0.68; p=0.05) 2. Additive interaction between HLA-A2 & IM was not significant 3.Additive interaction between *HLA-DR15*& anti-

			EBNA1 IgG (domain 385-420) was significant (p=0.05) 4. Additive interaction between HLA-A2 & anti-EBNA1 IgG (domain 385-420) was significant (p<0.001)
De Jager[62]& colleagues (2008)[d]	Cohort study, 148 female MS & 296 controls	EBV infection & DR15	Multiplicative interaction between *HLA-DR15*& anti-EBNA IgG was not significant (p=0.19)
Simon[64]& colleagues, (2010)[e]	Pool data from 3 studies, 442 MS & 865 controls	EBV infection & DR15	Multiplicative interaction between *HLA-DR15*& anti-EBNA was not significant (p=0.95) Multiplicative interaction between anti-EBNA IgG and smoking was significant (p=0.001) Estimates of OR of MS for increasing EBNA1 IgG titre: Never smokers: OR 1.8, 95% CI: 1.4-2.3; p<0.001 Ever smokers: OR 3.9, 95% CI: 2.7-5.7; p<0.001
Salzer[72]& colleagues (2013)[f]	Case-control, 192 MS & 384 controls	EBV infection & Low 25(OH)D	Interaction between low 25(OH)D & anti-EBNA IgG was not significant
Sundqvist[61]& colleagues, (2012)[g]	Case-control, 552 MS & 625 controls	EBV & smoking	Multiplicative interaction between anti-EBNA1 IgG and smoking was not significant (p=0.41) Estimates of OR of MS for increasing anti-EBNA1 IgG: Never smokers: OR 1.97, 95% CI: 1.39-2.80; $p=2x10^{-4}$ Ever smokers: OR 1.61, 95% CI: 1.16-2.23; $p=4x10^{-3}$
Salzer[73]& colleagues, (2013)[h]	Case-control, 192 MS & 384 controls	EBV & smoking	Multiplicative interaction between anti-EBNA1 IgG and cotinine was not significant (p=0.72) Estimates of OR of MS for increasing anti-EBNA1 IgG: Low cotinine group: OR 1.9, 95% CI: 1.2-3.0 High cotinine group: OR 2.1, 95% CI: 1.2-3.7

Abbreviations: FCD, first clinical diagnosis of central nervous system demyelination; SI, synergy index; AP, attributable proportion; MS, multiple sclerosis; IM, infectious mononucleosis; AP, attributable proportion; CI, confidence interval.

a population-based incident cases (diagnosed with McDonald criteria) and matched controls from the general population, a number of potential and established risk factors were analysed
b pooled data from two case-control studies, sufficient sample size. Rothman's causation model was used and supported the causal relationship between IM, HLA-DR15 and MS onset
c all incident patients were diagnosed with McDonald criteria, and recruited from 35 hospitals from 2005-2008
d prospective cohort study, sufficient sample size, patients were diagnosed with the Poser criteria
e pooled analysis from three investigations
f/h incident cases, samples were collected before disease onset
g clinic-based incident cases and matched controls from the general population, sufficient sample size

3.4.7 EBV and MS pathology

In general, evidence of EBV in the brains of MS patients could be strongly indicative of the involvement of EBV in the pathogenesis of MS. EBV-encoded small nuclear mRNA (EBER) has been found in brain of MS patients, particularly in areas of active disease, but was not detected in samples from other neurological patients[74]. In addition, the frequency of EBV-infected B-cells was associated with higher inflammation as quantified by $CD20^+$, $EBER^+$, and $CD8^+$ cells in MS brains. However, conflicting results were found in other studies wherein EBV markers could not be detected in post-mortem brain tissues from MS patients[75-78]. A previous review[79] discussed the contradiction of the results and suggested that the lack of sensitivity and specificity in EBV detection method was responsible for the discrepancies between studies. A more sensitive radioactive *in-situ* hybridisation technique with EBV-encoded small RNAs probes was utilised to mitigate these methodological limitations, confirming the existence of EBV-infected B-cells in MS lesions[80]. Higher expression of interferon-α and EBERs were found in active lesions of MS patients but not in inactive lesions or normal white matter, which suggested that activation of an innate immune response through latent EBV infection may contribute to the pathogenesis of MS. EBERs were also found in CNS lymphoma and stroke cases, which suggested a generalised association of EBV infection and CNS inflammation[81].

3.4.8 Mechanisms underlying EBV in MS

Various mechanisms have been proposed for the link between EBV and MS. Of these, perhaps the most plausible is molecular mimicry, where antigenic similarities between viral peptides and host epitopes could lead to autoimmune attack on host cells and

components. In support of this hypothesis, cross-reactivity between EBV antigens and myelin protein by cytotoxic T-lymphocytes has been demonstrated[82 83]. The property of cross-reactivity by T-lymphocytes also indicated that MS pathogenesis may include a T-cell-mediated autoimmune response in the central nervous system. However, aggregations of B-cells in the meninges of MS patients has challenged the idea that MS is solely a T-cell-mediated disease[74]. In comparing cerebrospinal fluid and serum samples from MS patients with tissues from patients with other neurological diseases, significantly higher concentrations of B-cells, but not T-cells, were found[84]. Based on these data, it was suggested that a dysfunctional reaction to EBV-infected B-cells by cytotoxic T lymphocytes leads to an EBV-infected B-cell expansion. Supporting this T and B-cell interrelationship in disease aetiology, Serafini and colleagues demonstrated that EBV-specific cytotoxic T-cells were increased along with increased numbers of B-cells in the brains of MS patients[74]. Pender and colleagues[85] found a decreasing frequency of $CD8^+$ T-cells reactive to EBV antigens in MS patients when compared to healthy controls. These results support the hypothesis[86] that a genetically determined deficiency of cytotoxic $CD8^+$ T-cells (responsible genes are located in the HLA complex[87]) could be responsible for an inability to control/suppress EBV-infected B-cells. These infected B-cells could then move into the CNS and produce pathogenic autoantibodies, as well as co-stimulatory survival signals that could inhibit the apoptosis of autoreactive T-cells.

3.4.9 Assessing the evidence for a relationship between EBV infection and MS: the Bradford-Hill criteria

When studying infectious agents that are specific to humans or which are difficult to culture in the laboratory, applying Koch's postulates to explore the causal relationship

is difficult. Therefore, Sir Austin Bradford-Hill's criteria would be adopted to examine the role of EBV on MS onset[88]. These criteria comprise 9 elements: 1) Strength – the strength of the association between EBV infection and MS onset was appreciable and clinically meaningful, MS patients showed a higher seropositivity of immune response to EBNA (OR=4.5; p<0.001), VCA (OR=4.5; p<0.001) and IM (OR=2.17; p<0.001) than controls[20 31]; 2) Consistency - the association between EBV infection and MS onset was constant across variable settings and in different populations. Using different biomarkers of EBV infection (EBNA or VCA) and EBV dependent IM both strongly supported the consistency of the association between EBV and MS. Sero-positivity of EBNA1 was associated with an increased risk of MS in different ethnic groups (whites, blacks and Hispanics)[89]; 3) Specificity –As previous discussion, contemporary view of specificity allows EBV to be the cause of MS and some other diseases like infectious mononucleosis and Hodgkin's lymphoma. The potential causal role of EBV in systemic lupus erythematosus[90] has opened the plausibility for another situation: considering specificity as to the induction of inflammation or unregulated immunological recognition, an argument for specificity between EBV infection status and MS (as a representative of autoimmune response) can be made. EBV was not associated with other diseases randomly, it was associated with MS but not other neurological dysfunction like stroke or Alzheimer; 4) Temporality - prospective cohort studies have demonstrated that higher immune response to EBV could be tested prior to MS onset. These studies, using differing methods in different populations and regions strongly inferred an important contribution of timing of aberrant immune response to EBV and subsequent risk; 5) Biological gradient - evidence to support a dose–response relationship between

immune response to EBV and MS risk arises from a range of cohort or case-control studies evaluating EBNA or VCA concentrations prior to and at the time of clinical onset of MS; 6) Plausibility - several theories have explained the potential causal mechanisms of EBV on MS, such as the molecular mimicry hypothesis, direct infection hypothesis, and the immune dysregulation hypothesis; 7) Coherence - in line with the epidemiological findings, a more predominant MS-like pathological features occurred when experimental autoimmune encephalomyelitis (EAE) model was infected with the murine homolog to EBV (γ-herpesvirus 68)[91].8) Experiment – no available method to prevent EBV infection is available to date, however, some treatment targeting the B-cells implied the role of EBV infection in MS onset as EBV could regulate the differentiation and function of memory B cells[92-94]. 9) Analogy – the causal role of EBV in Hodgkin lymphoma has been confirmed, and some research has discovered a genetic overlap between the EBV-related Hodgkin lymphoma and MS[95 96]. Thus, the analogy criterion was somewhat met."

Notably, causal role of EBV infection does not need all MS patients to be seropositive, because EBV could be one underlying cause in only a subset of cases. Association between the hepatitis B virus and liver cancer could support this statement: as patients without hepatitis B virus infection could also suffer from liver cancer, but the risk of liver cancer increased 50- to 100-fold when compared patients with and without infection[97]. It would be easier to interpret with the Rothman's pie model: onset of MS requires a complex interaction of many risk factor, but to complete an intact pie does not mean the occurrence of all risk factor. Therefore, some patients did not a show a higher immune response to EBV infection.

3.4.10 Conclusions regarding EBV & MS

Current epidemiology research has determined the aetiological role for EBV in MS onset; a prerequisite for developing MS with almost all cases of MS, and laboratory studies also support this conclusion. However, the association between immune responses to EBV and MS progression has not yet been confirmed.

3.5 Human Herpesvirus 6

3.5.1 What is human herpesvirus 6?

Another member of the herpesvirus family with a strong association with MS is human herpesvirus 6. HHV6 was first discovered in 1986[98], and the primary infection is generally asymptomatic. HHV6 is also a double-stranded DNA virus and can stay latent after the initial infection. Like EBV, anti-HHV6 IgG indicates a history of HHV6 infection, while anti-HHV6 IgM and HHV6 DNA are indicative of ongoing infection. Unlike EBV, HHV6 is comprised of two serotypes, HHV6A and HHV6B, each of which has distinct biological and immunological features[99]. Both types of HHV6 are neurotropic; however, a greater neurotropism by HHV6A than HHV6B[100] results may indicate a closer link between HHV6A and MS[101 102]. As with EBV, HHV6 infection is ubiquitous in most populations, suggesting interplay between HHV6 and other risk factors to modulate susceptibility to MS.

3.5.2 HHV6 biomarkers and MS risk

Some studies have demonstrated significantly higher frequencies of positivity and higher anti-HHV6 Ig titres in serum from MS patients than controls[103-105]. However, similarity of the serum anti-HHV6 antibody levels between MS patients and matched

controls in other studies has led to difficulty in determining the role of HHV6 in MS onset[106-110]. Moreover, unlike EBV, no researcher has designed prospective studies to demonstrate whether anti-HHV6 immune response increased prior to MS onset, which would support the temporality of the association. Direct detection of anti-HHV6-IgG in cerebrospinal fluid is strong evidence of HHV6's involvement in MS aetiology, and a higher prevalence of anti-HHV6 CSF IgG detection in MS patients when compared to other neurological disease patients (34% vs. 12%, p=0.05) has been demonstrated[110]. This study did not account for possible confounders and the sample size was low, but the comparison between MS patients (28 RRMS &10 progressive-MS) and patients with other neurological diseases (n=5) or inflammatory neurological diseases (n=21) still supported the association between HHV6 and MS pathogenesis. Because anti-HHV6 IgG could also cross react with other viruses, this finding could not exclude the impact of polyspecific B-cell reactions. A recent study[111] found that 10 peptides (eluted from CSF IgG columns) from 3/8 PPMS and 1/7 RRMS were in perfect homology with the major capsid protein of HHV6A, supporting the effect of HHV6 on MS pathology from the perspective of molecular mimicry. Small sample size and recruitment of all patients from one single centre could have influenced the validity and generalisability of the evidence.

In addition to anti-HHV6 IgG, anti-HHV6 IgM has been investigated for its association with MS aetiology, but again, the results have been inconsistent [106 112]. For example, using a rapid culture assay in serum, active HHV6 infection was present in 22 of 41 MS patients and none of 61 healthy controls ($p<0.001$)[113], while another study[106] found anti-HHV6-IgM was negative in CSF of all MS patients and only one

patient was positive in serum. Differences in results between studies may be due to different sample sizes, techniques used, or thresholds defining a positive result[108 114].

Detection of HHV6 DNA by PCR is a way to quantify latent virus in cells and free virus in serum or CSF. One case-control study with 78 MS patients and 123 healthy controls found a higher positivity of cell-free virus in MS patients than healthy controls[115] (60.2% vs. 14.6%; $p<0.001$). However, two other studies did not detect HHV6 DNA in serum, leading to their concluding that HHV6 DNA is not correlated with risk of MS[116 117]. A limitation of DNA detection method is that two samples from the same individual could exhibit different outcomes, given as viral reactivation could be occurring in the CNS but not the peripheral circulation, or vice versa, or latent virus could be present at quantifiable levels in one but not the other of these sites. This may explain why HHV6 DNA detection results have been inconsistent. The conflicting epidemiological research outcomes of the association between HHV6 and MS risk could indicate that there is no direct causal link between HHV6 infection and MS risk, or it may be that indirect mechanisms as seen with EBV may be involved, for example by gene-environment interaction.

3.5.3 HHV6 biomarkers and MS clinical course

Using data from a longitudinal study in Tasmania, our group previously found a strongly dose-dependent association ($p=0.001$) between anti-HHV6 IgG titre and subsequent risk of relapse[52]. HHV6 reactivation, as measured by anti-HHV6 IgM and HHV6 DNA, was rare, however, with anti-HHV6 IgM being detectable in only six samples from one individual out of 1,050 samples collected over nearly three years, and no association was observed between HHV6 DNA detection and either relapse or

disability[41]. A higher positivity of HHV6 DNA was detected at time of relapse when compared to remission (p=0.008)[118], reported from another longitudinal study following 59 participants for five months. When testing HHV6 DNA in CSF, increasing load was associated with elevated numbers of contrast-enhancing lesions on MRI[119]. A recent study demonstrated that anti-HHV6-IgG titre reached its highest levels approximately two weeks prior to relapse (p=0.01), this interval increasing to one month for anti-HHV6 IgM (p=0.03)[120]. This finding supported the association of anti-HHV6 and disease activity from temporal relationship. Current research suggests that immunological reaction against HHV6, either directly or indirectly, should be regarded as a potential risk factor in the progression of MS.

3.5.4 HHV6 and MS pathology

Detecting HHV6 directly in the brains of patients is strong evidence for the involvement of HHV6 in the pathogenesis of MS. With the assay of post-mortem samples in 7 MS patients and 3 patients with other disorders, using a fluorescent *in-situ* hybridisation technique, a higher level of HHV6 gene transcription was detected in MS patients, as well as in demyelinating lesions compared with normal-appearing white matter in samples from MS patients[121]. Another important finding in this study was that HHV6 gene transcription was restricted to oligodendrocytes, the myelin-producing glial cells of the CNS. Similar studies also found the frequency of HHV6 DNA was higher in lesions from MS patients than in normal-appearing white matter[113 122]. In contrast, another study found no difference in HHV6 detection in brains between MS patients and healthy donors[123].

3.5.5 Potential mechanisms of HHV6 association with MS

Molecular mimicry between HHV6 and host myelin components has been hypothesised to contribute to the pathology of MS. Another study found that seven residues were identical between myelin basic protein (MBP) and U24, a protein produced by HHV6[124]. Moreover, more than 50% of T-cells recognising MBP epitopes could be activated by HHV6 U24. This cross-reactivity was subsequently confirmed to be higher in MS patients than healthy controls or patients with other neurological diseases[125]. Another study found that HHV6 was capable of infecting glial precursor cells, resulting in alteration of cell morphology and impairment of cell replication[126]. This finding demonstrated that HHV6 has the potential to impact the production of myelin directly.

3.5.6 Conclusions regarding HHV6 & MS

The role of HHV6 in MS onset is elusive, with conflicting results from multiple case-control and cohort studies[41 127 128]. Additional longitudinal cohort studies, ideally measuring both serological and viral load parameters in both CSF and peripheral circulation, are needed to determine whether a true association exists. The role of abnormal immune response to HHV6 in the disease activity of MS has been supported by an increasing number of cohort and laboratory studies. Interestingly, the effect of HHV6 in MS is at least partly in contrast to EBV, where most research has demonstrated the essential role in MS onset other than disease activity. The association between HHV-6 and multiple sclerosis primarily relied on pathological studies, observational studies were scarce and could not provide sufficient evidence from the epidemiological perspective. To prove causation, diverse research

methodologies are required. Notably, publication bias may influence our interpretation of the results, and sample size of current research was still small. Therefore, conclusions about the role of HHV6 on MS clinical course are not yet able to be drawn.

3.6 Human endogenous retroviruses and MS

Although this thesis did not perform the analysis between HERVs and clinical course of MS, mounting evidence suggested that HERVs are associated with MS onset and progression. Therefore, we will briefly review the roles of HERVs in MS.

3.6.1 What are human endogenous retroviruses and multiple sclerosis-associated retrovirus?

Endogenous retroviruses originate from ancestral exogenous infecting viruses which were incorporated into the host genome and entered the germ line during evolution. Endogenous retroviruses make up nearly 8% of the human genome[129]. While through the process of evolution most endogenous retroviruses have become incomplete and lost their function, open reading frames still exist in some HERVs which encode functional proteins. While no longer capable of replication and lysis of host cells, some viral components, including *gap, pro, pol,* and *env* genes, are still transcriptionally active. Some of these have become useful in host function; for instance, the HERV-derived synctin-1 protein is produced in the placenta and plays a role in placental implantation[130]. However, neurotoxic effects of some HERV-produced proteins expressed in the CNS led to the hypothesis that they have a role in MS onset. Of the various HERVs, one, aptly called Multiple Sclerosis-associated Retrovirus (MSRV), is of particular interest in MS. MSRV was first discovered within

leptomeningeal cells isolated from the CSF of MS patients and found to possess reverse-transcriptase activity[131]. Evaluation of the sequence of MSRV showed close relatedness with the HERV-W family. Frequent detection of MSRV in the brains of MS patients has supported its potential role in MS aetiology[132].

3.6.2 HERV biomarkers and MS risk

Measurement of DNA copy number or transcription levels or expression of HERVs *env* or *pol* genes in MS patients could aid in understanding the association between HERVs and MS. Several case-control studies have demonstrated a higher positivity of MSRV RNA in serum of MS patients than healthy controls[133 134]. For instance, a multicentre study with 149 MS patients and 153 controls found that the positivity of MSRV *pol* gene was 71.4% in MS patients, compared to only 17.3% in healthy controls[135]. Using fluorescence *in-situ* hybridisation (FISH) and PCR, DNA copies of MSRV *pol* were found to be higher in PBMCs of 16 MS patients when compared with 10 healthy controls[136]. Notably, the quality of the evidence in this study were influenced by 1) all cases were prevalent with the durations of disease ranged from 2 to 174 months, and all controls were healthy individuals with no information about the match degree; 2) not controlled for possible confounders; 3) sample size was low. Garcia-Montojo and colleagues[137] found higher levels of MSRV transcription in PBMCs from MS patients than controls. Moreover, the MSRV RNA level was higher in SPMS patients than RRMS. The same group also found that the copy number of MSRV was higher in MS patients than healthy controls ($p=4.15x10^{-7}$)[138].

HERV-Fc1 belongs to the HERV H/F family, and MS patients have increased levels of HERV-F *gap* or *env* expression in PBMCs compared with controls[139 140]. The

HERV-Fc1 RNA load was four-fold higher in plasma from MS patients compared with those from healthy controls[139]. SNP rs3802981, located in the first intron of the restriction gene *TRIM5*, is able to control the transcription of HERV-Fc. An inverse association[141] was found between the T-allele of SNP rs3802981and MS risk (OR=0.59, p=0.002). This study also found that C-allele of SNP rs391745 (near HERV-Fc1) was more common in MS patients (OR=1.54, $p=1.3x10^{-5}$). A subsequent study showed that C-allele of rs391745 was more common among MS patients with relapse-onset than progressive-onset[142]. This association was supported by a pooled analysis, yielding an OR of 1.27 (95% CI: 1.11-1.45)[143]. Other HERVs such as HERV-k[144 145], have shown inconsistent associations with MS onset, however.

3.6.3 HERV biomarkers and MS clinical course

Studies investigating the association between HERVs and MS clinical course have been few and inconsistent. One study (n=112) demonstrated that the level of HERV-W transcription was significantly associated with the Multiple Sclerosis Severity Score (MSSS) in female patients (r=0.34, p=0.017), as well as showing a weaker and non-significant trend (r=0.22, p=0.16) in males[137]. In addition, the number of DNA copies of *HERV-W* on chromosome Xq22.3 and the polymorphisms of rs6622189, located in the env region of *HERV-W*, were associated with MSSS score in women. Since this gene is located on the X chromosome, to some extent this may be relevant in the well-known female preponderance in MS[146]. Using RT-qPCR, one case-control study with 178 MS patients found that a higher DNA copy number of MSRV in PBMCs was associated with an increasing EDSS (4.92 vs. 2.83; p=0.032) and MSSS (6.40 vs. 3.92; p=0.044) in women[138]. In this study, patients and control were not 1:1

matched (124 controls) and some important confounders were not controlled for, which may influence the validity of the findings. One six-year prospective study with 18 MS patients found that, despite a similar disability and relapse rate at early stages of follow-up, those with detectable MSRV in their CSF at baseline had significantly higher EDSS scores (4.3 vs. 2.2; p=0.004) and annual relapse rate (0.5 vs. 0.3; p=0.01)[147] by the end of follow-up. Overall, although the data is insufficient at this stage to conclude that a higher HERV copy number or transcription rate is associated with disease progression, HERV biomarkers should be regarded as potential risk factors predicting the progression of MS.

3.6.4 HERV and MS pathology

Repeated isolation of MSRV from the leptomeninges or choroid plexus[131 132] has indicated the role of HERVs in MS pathogenesis. Concentrations of several HERV markers were higher in lesions of MS patients when compared to non-lesion samples from the same patients or from brain tissues of matched controls. RNA levels of HERV-W were higher in lesions of MS patients than in lesions of other neurological diseases or white matter of healthy controls[123 148]. Testing 8 MS patients, e*nv* immunoreactivity was found in activated lesions but absent in normal-appearing white matter[149]. In keeping with this study, antibodies to the *env* protein of HERV-W could only be detected in lesions of MS patients[123]. Similarly, accumulation of *gag* antigens from HERV-W were detected in axonal lesions in MS patients[150] when compared to non-MS control brains. However, using RT-PCR one study found no significant difference in HERV-W transcription levels in white matter between MS patients and healthy controls[151]. Methodological difference may explain the conflicting findings;

however, most studies to date demonstrated a higher frequency of HERVs transcription or expression in lesions of MS patients, which suggested the potential association between HERVs and MS pathology.

In addition to a direct impact of HERV products, some studies have investigated whether MSRV-env protein could directly modulate the secretion of cytokines. An increasing frequency of interferon-γ and tumour necrosis factor-α (TNF-α) and decreased frequency of interleukin-10 from MSRV-env simulated PBMCs have been shown when compared 13 MS patients in relapse with 17 MS patients in remission[152]. A similar study found an increased secretion of IL-6 and TNF-α by MSRV-env simulated PBMCs when cultured in-vitro[153]. In addition, animal experiments demonstrated that *env* protein from MSRV could induce the synthesis of proinflammatory cytokines such as interferon-γ and TNF-α [154]. The study's cross-sectional design using prevalent MS patients may decrease the validity of the findings; however, HERVs tests are costly at the present stage, meaning studies with large sample size are logistically challenging. Prospective study designs are less important for HERVs studies due to its genetic nature. All these results suggest that MSRV *env* particles could work as a superantigen, affecting T-cells and causing overexpression of proinflammatory cytokines. With similar characteristics to a superantigen, MSRV-*env* could trigger aberrant immune response with abnormal polyclonal T-lymphocyte activation[153]. Recent studies[155] [156] also suggested that MSRV *env* can activate toll-like receptor 4 which can lead to the secretion of proinflammatory cytokines.

Despite its utility in the placenta, studies have demonstrated increased levels of synctin-1 in MS lesions, especially in glial cells[157]. Endoplasmic reticulum stress could be induced by synctin-1[158] and one of the chaperones, old astrocyte specifically induced substance (OASIS), was capable of strengthening the expression of inducible nitric oxide synthase (iNOS), resulting in an overexpression of free radicals. This may be relevant to MS pathogenesis, particularly as oligodendrocytes are especially vulnerable to damage mediated by free radicals.

Env protein of *HERV-k18* is regarded as a superantigen that can stimulate T-cells and elicit a powerful immune response. EBV-infected B lymphocytes are capable of transactivating the *env* gene of *HERV-k18*[159], and subsequent studies[160] have revealed that latent membrane protein (LMP) plays an essential role in the induction of the transactivation. Experiments using murine B lymphocytes has demonstrated that immunoreceptor tyrosine-based activation motif (ITAM) of LMP-2A was capable of initiating a signalling cascade and induction of *HERV-k18 env*[161].

3.6.5 Conclusions regarding HERV/MSRV & MS

Through the consistent detection of HERV biomarkers, the evidence for HERVs in MS onset is appreciable. However, without evidence from prospective cohort studies, particularly the transcription or expression level of HERVs prior to MS onset is less clear. Also, a clear pathway for biological plausibility has yet to be fully elucidated. Further work is required to clearly demonstrate that HERVs may influence MS onset or clinical course. These will require both epidemiological associations in cohort studies and basic science experiments.

3.7 Potential therapies that could influence the immune response to EBV/HHV6/HERVs in MS patients

Given the diverse evidence from epidemiology and laboratory research implicating the role of human herpesvirus infection(s) in MS, it has been assumed that efforts to prevent viral infection by vaccination or other prophylactic interventions could reduce MS risk or moderate its clinical course. Despite the tremendous potential for this point of intervention in MS prevention and treatment, relatively few studies aimed at treating MS from the perspective of viral infection have been conducted. To date, the most notable experiments were three phase-3 clinical trials (OPERA I, OPERA II and ORATORIO study) of ocrelizumab, a humanised anti-CD20 antibody, which selectively removes mature B-cells based on the hypothesis that dysregulated B-lymphocytes are involved in MS pathogenesis. These studies demonstrated patients administered with therapy showed a lower relapse rate and recovery from relapses in RRMS and moderate but still statistically significant effects on PPMS progression. The period of follow-up was still too short to conclude its effects on long-term disability progression, but these trials suggested the significant role of removing CD20-positive B-cells on the reduction of the number of EBV-infected B-cells, and may be one of the mechanisms by which this medication affects MS clinical course. Antiviral drugs like acyclovir[162] and valacyclovir[163] are nucleoside-based medications that inhibit the function of viral DNA polymerase and block lytic replication after being taken up by the infected B-cells. These have been tested in clinical trials but neither reached statistical significance in reducing exacerbation rate or number of new active MRI-detected lesions. However, as shown above, there is little evidence that viral replication and cell lysis are the drivers of MS onset or progression. If in fact

another mechanism is at play, including potential transactivation of HERVs or molecular mimicry-induced autoimmune response, then the impacts of these viruses on MS will not be influenced by these antiviral drugs.

Some novel forms of clinical research have also been tested. For example, according to the theory that exhaustion of EBV-specific CD8+ T-cells are associated with MS pathogenesis, supplementation of EBV-specific CD8+ T-cells was performed in one SPMS patient and this patient showed decreased clinical activity on MRI and reduced intrathecal immunoglobulin production[47]. Natalizumab, is a humanised anti α4-integrin monoclonal antibody therapy that prevents leukocyte migration into the central nervous system[164] and could interfere with immunoglobulin synthesis[165], was tested for its influence on antibodies' level to human herpesvirus infection. Recently, a two-year follow-up longitudinal study showed a decreased level of anti-HHV6-IgM/IgG in natalizumab-treated patients and a correlation between the variation of antibodies and the clinical response to treatment[120]. This study also found that patients with interferon-βand glatiramer acetate showing no significant reduction of anti-HHV6 IgG. This indicates the potential to influence MS disease course, which aligns with the studies that observed an association between anti-HHV6-IgG and relapse. Another prospective study with 21 months follow-up in 21 MS patients found that anti-EBNA1 was stable during the treatment period[48], which may suggest that inactive EBV was not associated with MS clinical course.

Anantti-EBV vaccine to prevent EBV infection has not been developed for market yet. Some research has attempted to develop a vaccine targeting viral glycoprotein 350, which is the main antigen targeted by the immune system. Unfortunately, the vaccine

was not effective at preventing EBV infection[166]. Moreover, some research has indicated that EBV infection during childhood is protective, so we could not exclude the possibility that being infected with EBV is developing other forms of immune tolerance.

GNbAC1, a humanised monoclonal antibody targeted at HERV-env has been tested in a phase II clinical trial and showed a significant reduction of HERV transcripts in the treated group[167]. Conclusions about the clinical benefits could not be drawn yet since the observation period was only six months, but only one SPMS patient developed a new lesion on MRI and the other 9 patients were clinically stable. Some laboratory data also demonstrated that GNbAC1 could prevent MSRV-env mediated proinflammatory gene transcription and restore differentiation of oligodendrocytes precursor cells [168].

3.8 Conclusions

A large number of epidemiological studies have ascertained the association between EBV and MS risk. The evidence for causality is well-supported within the present methodological framework to the extent that observational epidemiological outcomes can ascribe causality. Discrepancies in post-mortem and laboratory investigations of the role of EBV in MS patients are probably due to the diversity of sample quality and detection techniques, with most research inclined to support this association.

Owing to the lack of well-powered prospective cohort study, evidence for HHV6's role in MS risk is not as robust as that for EBV. However, an increasing number of studies support a potential role in MS clinical course. HHV6 can be regarded as a

potential risk factor for MS progression, but clearly there is more work required to confirm the link. Interesting, the role of EBV and HHV6 are somewhat opposed to each other: EBV is strongly associated with the onset of MS while there is more evidence that HHV6 is associated with disease activity. However, the insignificant association between EBV infection and MS disease activity in current epidemiological studies may suggest that biomarkers like anti-EBNA or anti-VCA are not suitable for studies of MS clinical course (Figure). Future studies focusing on novel biomarkers may reveal a closer connection to the immune response to EBV. Moreover, future studies should clarify the variation of anti-EBV after the use disease modifying therapy in MS patients.

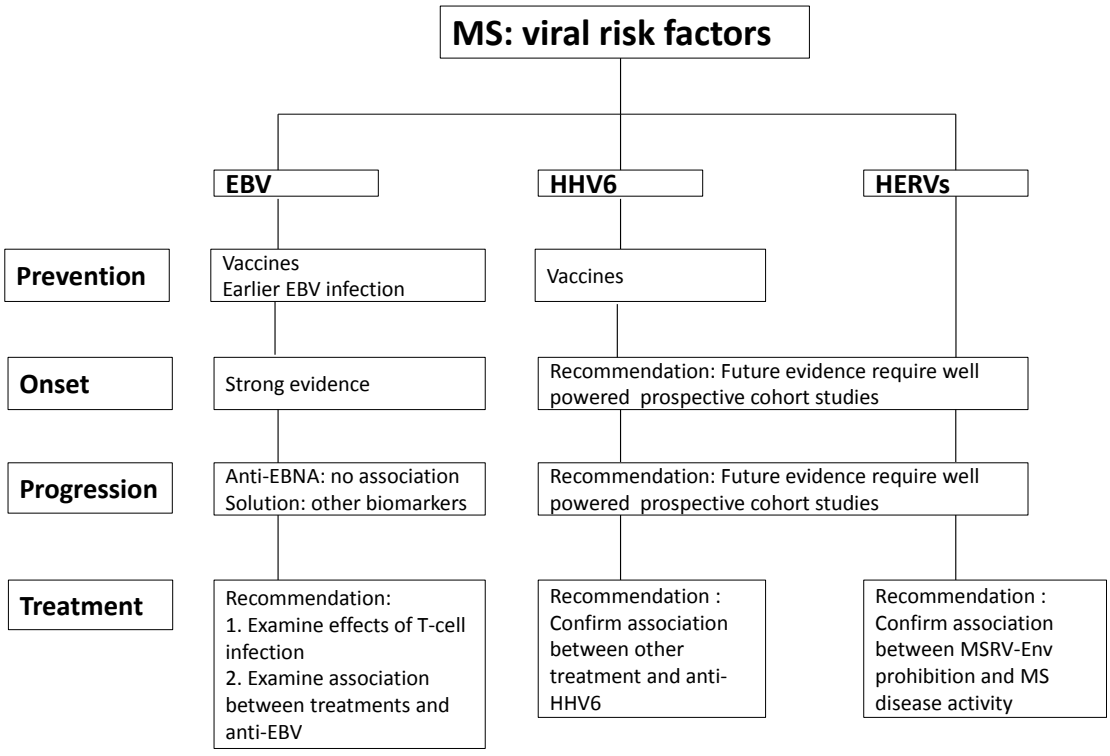

Figure 3.1Recommendation for the future studies of the role of EBV, HHV6 and HERVs in MS onset, progression and treatment.

In terms of HERVs, the neurotoxic role and mechanisms of MSRV-env were supported by laboratory findings and some clinical pilot trials have been performed from this perspective. Epidemiologically, while there is some preliminary evidence from case-control studies linking HERV to MS onset and clinical course, further prospective cohort studies are needed to substantiate these results and exclude the possibility of reverse causality.

If viral infection, principally EBV infection, was an essential part of MS pathogenesis, prevention of EBV infection by vaccination could be a feasible approach. Similarly, novel studies assessing the role of immunomodulation of the complex EBV induced changes within the MS patient may provide another avenue for treatment. The exciting finding of the success of B-cell depletion in RRMS and PPMS adds further weight to the role of B-cell dysregulation possibly from HHV infection as a major driver of MS clinical course. Future studies aimed at understanding the mechanism and role of viral infection on the MS onset and progression will hopefully identify advanced methods for the prevention and treatment of MS.

3.9 Postscript

This chapter demonstrated the role of viral infection on MS onset and progression, and we found that EBV was associated with MS onset but the effects on MS progression were uncertain. In contrast, a number of studies demonstrated that higher immune response to HHV6 infection were associated with clinical course in MS patients. The next chapter is about the association between latitude and age at onset.

3.10 Reference

1. Mechelli R, Annibali V, Ristori G, et al. Multiple sclerosis etiology: beyond genes and environment. *Expert review of clinical immunology* 2010;6(3):481-90. doi: 10.1586/eci.10.11
2. Ramagopalan SV, Dobson R, Meier UC, et al. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet neurology* 2010;9(7):727-39. doi: 10.1016/S1474-4422(10)70094-6
3. Haines JL, Terwedow HA, Burgess K, et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group. *Human molecular genetics* 1998;7(8):1229-34.
4. Rubio JP, Bahlo M, Butzkueven H, et al. Genetic dissection of the human leukocyte antigen region by use of haplotypes of Tasmanians with multiple sclerosis. *American journal of human genetics* 2002;70(5):1125-37. doi: 10.1086/339932
5. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. *Lancet Neurology* 2010;9(6):599-612. doi: 10.1016/s1474-4422(10)70086-7 [published Online First: 2010/05/25]
6. Handel AE, Williamson AJ, Disanto G, et al. Smoking and multiple sclerosis: an updated meta-analysis. *PloS one* 2011;6(1):e16149. doi: 10.1371/journal.pone.0016149
7. Christensen T. Human herpesviruses in MS. *International MS journal / MS Forum* 2007;14(2):41-47.
8. Christensen T. HERVs in neuropathogenesis. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 2010;5(3):326-35. doi: 10.1007/s11481-010-9214-y [published Online First: 2010/04/28]
9. Nystad TW, Myrmel H. Prevalence of primary versus reactivated Epstein-Barr virus infection in patients with VCA IgG-, VCA IgM- and EBNA-1-antibodies and suspected infectious mononucleosis. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2007;38(4):292-7. doi: 10.1016/j.jcv.2007.01.006 [published Online First: 2007/03/06]
10. Henle W, Henle G, Andersson J, et al. Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 1987;84(2):570-4.
11. Bornkamm GW, Hammerschmidt W. Molecular virology of Epstein-Barr virus. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 2001;356(1408):437-59. doi: 10.1098/rstb.2000.0781
12. Odumade OA, Hogquist KA, Balfour HH, Jr. Progress and problems in understanding and managing primary Epstein-Barr virus infections. *Clinical microbiology reviews* 2011;24(1):193-209. doi: 10.1128/cmr.00044-10 [published Online First: 2011/01/15]
13. Savoldo B, Cubbage ML, Durett AG, et al. Generation of EBV-specific CD4+ cytotoxic T cells from virus naive individuals. *Journal of immunology (Baltimore, Md : 1950)* 2002;168(2):909-18. [published Online First: 2002/01/05]

14. Cesarman E. Gammaherpesviruses and lymphoproliferative disorders. *Annual review of pathology* 2014;9:349-72. doi: 10.1146/annurev-pathol-012513-104656 [published Online First: 2013/10/12]
15. Pakpoor J, Disanto G, Gerber JE, et al. The risk of developing multiple sclerosis in individuals seronegative for Epstein-Barr virus: a meta-analysis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(2):162-6. doi: 10.1177/1352458512449682
16. Ascherio A, Munch M. Epstein-Barr virus and multiple sclerosis. *Epidemiology (Cambridge, Mass)* 2000;11(2):220-4. [published Online First: 2000/10/06]
17. Pakpoor J, Ramagopalan SV. Epstein-Barr virus is a necessary causative agent in the pathogenesis of multiple sclerosis: yes. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(13):1690-1. doi: 10.1177/1352458513506505 [published Online First: 2013/11/13]
18. Lucas RM, Hughes AM, Lay ML, et al. Epstein-Barr virus and multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2011;82(10):1142-8. doi: 10.1136/jnnp-2011-300174 [published Online First: 2011/08/13]
19. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Annals of neurology* 2006;59(3):499-503. doi: 10.1002/ana.20820
20. Handel AE, Williamson AJ, Disanto G, et al. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PloS one* 2010;5(9) doi: 10.1371/journal.pone.0012496
21. Nielsen TR, Rostgaard K, Nielsen NM, et al. Multiple sclerosis after infectious mononucleosis. *Archives of neurology* 2007;64(1):72-5. doi: 10.1001/archneur.64.1.72
22. McMichael AJ, Hall AJ. Does immunosuppressive ultraviolet radiation explain the latitude gradient for multiple sclerosis? *Epidemiology* 1997;8(6):642-5. [published Online First: 1997/11/05]
23. Ramagopalan SV, Handel AE, Giovannoni G, et al. Relationship of UV exposure to prevalence of multiple sclerosis in England. *Neurology* 2011;76(16):1410-4. doi: 10.1212/WNL.0b013e318216715e [published Online First: 2011/04/20]
24. Simpson Jr. SL, Blizzard L, Otahal P, et al. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *Journal of Neurology, Neurosurgery and Psychiatry* 2011;82(10):1132-41. doi: doi:10.1136/jnnp.2011.240432
25. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. *Neurology* 2008;71(2):129-35. doi: 10.1212/01.wnl.0000316802.35974.34
26. Disanto G, Pakpoor J, Morahan JM, et al. Epstein-Barr virus, latitude and multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(3):362-5. doi: 10.1177/1352458512451942
27. Warner HB, Carp RI. Multiple sclerosis and Epstein-Barr virus. *Lancet* 1981;2(8258):1290.
28. Grant W. Latitude and multiple sclerosis prevalence: vitamin D reduces risk of Epstein--Barr virus infection. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(3):373.
29. Lossius A, Riise T, Pugliatti M, et al. Season of infectious mononucleosis and risk

of multiple sclerosis at different latitudes; the EnvIMS Study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013 doi: 10.1177/1352458513505693 [published Online First: 2013/09/28]
30. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Annals of neurology* 2007;61(4):288-99. doi: 10.1002/ana.21117
31. Almohmeed YH, Avenell A, Aucott L, et al. Systematic review and meta-analysis of the sero-epidemiological association between Epstein Barr virus and multiple sclerosis. *PloS one* 2013;8(4):e61110. doi: 10.1371/journal.pone.0061110 [published Online First: 2013/04/16]
32. Ascherio A, Munger KL, Lennette ET, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA : the journal of the American Medical Association* 2001;286(24):3083-8. [published Online First: 2002/01/05]
33. Sundstrom P, Juto P, Wadell G, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. *Neurology* 2004;62(12):2277-82.
34. Levin LI, Munger KL, Rubertone MV, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. *JAMA : the journal of the American Medical Association* 2005;293(20):2496-500. doi: 10.1001/jama.293.20.2496
35. DeLorenze GN, Munger KL, Lennette ET, et al. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. *Archives of neurology* 2006;63(6):839-44. doi: 10.1001/archneur.63.6.noc50328
36. Levin LI, Munger KL, Rubertone MV, et al. Multiple sclerosis and Epstein-Barr virus. *Jama* 2003;289(12):1533-6.
37. Cocuzza CE, Piazza F, Musumeci R, et al. Quantitative detection of epstein-barr virus DNA in cerebrospinal fluid and blood samples of patients with relapsing-remitting multiple sclerosis. *PloS one* 2014;9(4):e94497. doi: 10.1371/journal.pone.0094497 [published Online First: 2014/04/12]
38. Villegas E, Santiago O, Carrillo JA, et al. Low intrathecal immune response of anti-EBNA-1 antibodies and EBV DNA from multiple sclerosis patients. *Diagnostic microbiology and infectious disease* 2011;70(1):85-90. doi: 10.1016/j.diagmicrobio.2010.11.013 [published Online First: 2011/03/11]
39. Lindsey JW, Hatfield LM, Crawford MP, et al. Quantitative PCR for Epstein-Barr virus DNA and RNA in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2009;15(2):153-8. doi: 10.1177/1352458508097920
40. Lucas RM, Ponsonby AL, Dear K, et al. Current and past Epstein-Barr virus infection in risk of initial CNS demyelination. *Neurology* 2011;77(4):371-9. doi: 10.1212/WNL.0b013e318227062a [published Online First: 2011/07/15]
41. Simpson S, Jr., Taylor B, Burrows J, et al. EBV & HHV6 reactivation is infrequent and not associated with MS clinical course. *Acta neurologica Scandinavica* 2014 doi: 10.1111/ane.12268 [published Online First: 2014/06/05]
42. Wandinger K, Jabs W, Siekhaus A, et al. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. *Neurology* 2000;55(2):178-

84.
43. Torkildsen O, Nyland H, Myrmel H, et al. Epstein-Barr virus reactivation and multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2008;15(1):106-8. doi: 10.1111/j.1468-1331.2007.02009.x
44. Buljevac D, van Doornum GJ, Flach HZ, et al. Epstein-Barr virus and disease activity in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2005;76(10):1377-81. doi: 10.1136/jnnp.2004.048504 [published Online First: 2005/09/20]
45. Farrell RA, Antony D, Wall GR, et al. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. *Neurology* 2009;73(1):32-8. doi: 10.1212/WNL.0b013e3181aa29fe [published Online First: 2009/05/22]
46. Angelini DF, Serafini B, Piras E, et al. Increased CD8+ T cell response to Epstein-Barr virus lytic antigens in the active phase of multiple sclerosis. *PLoS pathogens* 2013;9(4):e1003220. doi: 10.1371/journal.ppat.1003220 [published Online First: 2013/04/18]
47. Pender MP, Csurhes PA, Smith C, et al. Epstein-Barr virus-specific adoptive immunotherapy for progressive multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514521888 [published Online First: 2014/02/05]
48. Castellazzi M, Delbue S, Elia F, et al. Epstein-Barr Virus Specific Antibody Response in Multiple Sclerosis Patients during 21 Months of Natalizumab Treatment. 2015;2015:901312. doi: 10.1155/2015/901312
49. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Annals of neurology* 2001;50(1):121-7.
50. Lünemann JD, Tintoré M, Messmer B, et al. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Annals of neurology* 2010;67(2):159-69. doi: 10.1002/ana.21886
51. Munger KL, Fitzgerald KC, Freedman MS, et al. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. *Neurology* 2015;85(19):1694-701. doi: 10.1212/wnl.0000000000002099 [published Online First: 2015/10/11]
52. Simpson S, Taylor B, Dwyer DE, et al. Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis. *Multiple Sclerosis Journal* 2011;18(6):799-806. doi: 10.1177/1352458511428081
53. Makhani N, Banwell B, Tellier R, et al. Viral exposures and MS outcome in a prospective cohort of children with acquired demyelination. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(3):385-8. doi: 10.1177/1352458515595876 [published Online First: 2015/07/23]
54. Kvistad S, Myhr KM, Holmoy T, et al. Antibodies to Epstein-Barr virus and MRI disease activity in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514533843
55. Zivadinov R, Zorzon M, Weinstock-Guttman B, et al. Epstein-Barr virus is associated with grey matter atrophy in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2009;80(6):620-5. doi:

10.1136/jnnp.2008.154906
56. van der Mei I, Lucas RM, Taylor BV, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. *Mult Scler* 2015 doi: 10.1177/1352458515594040
57. Disanto G, Hall C, Lucas R, et al. Assessing interactions between HLA-DRB1*15 and infectious mononucleosis on the risk of multiple sclerosis. *Mult Scler* 2013 doi: 1352458513477231 [pii]10.1177/1352458513477231 [published Online First: 2013/02/16]

58. Nielsen T, Rostgaard K, Askling J, et al. Effects of infectious mononucleosis and HLA-DRB1*15 in multiple sclerosis. *Mult Scler* 2009

59. Sundqvist E, Sundstrom P, Linden M, et al. Epstein-Barr virus and multiple sclerosis: interaction with HLA. *Genes and immunity* 2012;13(1):14-20. doi: 10.1038/gene.2011.42 [published Online First: 2011/07/22]
60. Ramagopalan SV, Sadovnick AD, Ebers GC, et al. Effects of infectious mononucleosis and HLA-DRB1*15 in multiple sclerosis. *Mult Scler* 2010;16(1):127-8. doi: 16/1/127 [pii]10.1177/1352458509350313 [published Online First: 2010/01/07]

61. Sundqvist E, Sundstrom P, Linden M, et al. Lack of replication of interaction between EBNA1 IgG and smoking in risk for multiple sclerosis. *Neurology* 2012;79(13):1363-8. doi: 10.1212/WNL.0b013e31826c1ab7
62. De Jager PL, Simon KC, Munger KL, et al. Integrating risk factors: HLA-DRB1*1501 and Epstein-Barr virus in multiple sclerosis. *Neurology* 2008;70(13 Pt 2):1113-8. doi: 10.1212/01.wnl.0000294325.63006.f8
63. Sundstrom P, Nystrom L, Jidell E, et al. EBNA-1 reactivity and HLA DRB1*1501 as statistically independent risk factors for multiple sclerosis: a case-control study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(8):1120-2. doi: 10.1177/1352458508092353
64. Simon KC, van der Mei IA, Munger KL, et al. Combined effects of smoking, anti-EBNA antibodies, and HLA-DRB1*1501 on multiple sclerosis risk. *Neurology* 2010;74(17):1365-71. doi: 10.1212/WNL.0b013e3181dad57e
65. Sundstrom P, Nystrom M, Ruuth K, et al. Antibodies to specific EBNA-1 domains and HLA DRB1*1501 interact as risk factors for multiple sclerosis. *Journal of neuroimmunology* 2009;215(1-2):102-7. doi: 10.1016/j.jneuroim.2009.08.004 [published Online First: 2009/09/08]
66. Rubicz R, Yolken R, Drigalenko E, et al. A genome-wide integrative genomic study localizes genetic factors influencing antibodies against Epstein-Barr virus nuclear antigen 1 (EBNA-1). *PLoS genetics* 2013;9(1):e1003147. doi: 10.1371/journal.pgen.1003147 [published Online First: 2013/01/18]
67. Zhou Y, Zhu G, Charlesworth JC, et al. Genetic loci for Epstein-Barr virus nuclear antigen-1 are associated with risk of multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016 doi: 10.1177/1352458515626598 [published Online First: 2016/01/29]
68. Yenamandra SP, Hellman U, Kempkes B, et al. Epstein-Barr virus encoded EBNA-3 binds to vitamin D receptor and blocks activation of its target genes. *Cellular and molecular life sciences : CMLS* 2010;67(24):4249-56. doi:

10.1007/s00018-010-0441-4
69. Carlson NG, Rose JW. Vitamin D as a clinical biomarker in multiple sclerosis. *Expert opinion on medical diagnostics* 2013;7(3):231-42. doi: 10.1517/17530059.2013.772978
70. Ramien C, Pachnio A, Sisay S, et al. Hypovitaminosis-D and EBV: no interdependence between two MS risk factors in a healthy young UK autumn cohort. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(6):751-3. doi: 10.1177/1352458513509507 [published Online First: 2013/11/07]
71. Rosjo E, Lossius A, Abdelmagid N, et al. Effect of high-dose vitamin D3 supplementation on antibody responses against Epstein-Barr virus in relapsing-remitting multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016 doi: 10.1177/1352458516654310 [published Online First: 2016/06/22]
72. Salzer J, Nystrom M, Hallmans G, et al. Epstein-Barr virus antibodies and vitamin D in prospective multiple sclerosis biobank samples. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(12):1587-91. doi: 10.1177/1352458513483888 [published Online First: 2013/04/04]
73. Salzer J, Stenlund H, Sundstrom P. The interaction between smoking and Epstein-Barr virus as multiple sclerosis risk factors may depend on age. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013 doi: 10.1177/1352458513507820 [published Online First: 2013/10/11]
74. Serafini B, Rosicarelli B, Franciotta D, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *The Journal of experimental medicine* 2007;204(12):2899-912. doi: 10.1084/jem.20071030
75. Hilton DA, Love S, Fletcher A, et al. Absence of Epstein-Barr virus RNA in multiple sclerosis as assessed by in situ hybridisation. *Journal of neurology, neurosurgery, and psychiatry* 1994;57(8):975-6.
76. Opsahl ML, Kennedy PG. An attempt to investigate the presence of Epstein Barr virus in multiple sclerosis and normal control brain tissue. *Journal of neurology* 2007;254(4):425-30. doi: 10.1007/s00415-006-0316-7
77. Sargsyan SA, Shearer AJ, Ritchie AM, et al. Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis. *Neurology* 2010;74(14):1127-35. doi: 10.1212/WNL.0b013e3181d865a1
78. Willis SN, Stadelmann C, Rodig SJ, et al. Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. *Brain : a journal of neurology* 2009;132(Pt 12):3318-28. doi: 10.1093/brain/awp200
79. Lassmann H, Niedobitek G, Aloisi F, et al. Epstein-Barr virus in the multiple sclerosis brain: a controversial issue--report on a focused workshop held in the Centre for Brain Research of the Medical University of Vienna, Austria. *Brain : a journal of neurology* 2011;134(Pt 9):2772-86. doi: 10.1093/brain/awr197
80. Serafini B, Muzio L, Rosicarelli B, et al. Radioactive in situ hybridization for Epstein-Barr virus-encoded small RNA supports presence of Epstein-Barr virus in the multiple sclerosis brain. *Brain : a journal of neurology* 2013;136(Pt 7):e233. doi: 10.1093/brain/aws315 [published Online First: 2013/01/29]
81. Tzartos JS, Khan G, Vossenkamper A, et al. Association of innate immune

activation with latent Epstein-Barr virus in active MS lesions. *Neurology* 2012;78(1):15-23. doi: 10.1212/WNL.0b013e31823ed057
82. Holmoy T, Kvale EO, Vartdal F. Cerebrospinal fluid CD4+ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein. *Journal of neurovirology* 2004;10(5):278-83. doi: 10.1080/13550280490499524
83. Lunemann JD, Jelcic I, Roberts S, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *The Journal of experimental medicine* 2008;205(8):1763-73. doi: 10.1084/jem.20072397
84. Kuenz B, Lutterotti A, Ehling R, et al. Cerebrospinal fluid B cells correlate with early brain inflammation in multiple sclerosis. *PloS one* 2008;3(7):e2559. doi: 10.1371/journal.pone.0002559
85. Pender MP, Csurhes PA, Lenarczyk A, et al. Decreased T cell reactivity to Epstein-Barr virus infected lymphoblastoid cell lines in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2009;80(5):498-505. doi: 10.1136/jnnp.2008.161018
86. Pender MP. The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *Neuroscientist* 2011;17(4):351-67. doi: 10.1177/1073858410381531 [published Online First: 2010/11/16]
87. Ferreira M, Mangino M, Brumme C, et al. Quantitative trait loci for CD4:CD8 lymphocyte ratio are associated with risk of type 1 diabetes and HIV-1 immune control. *Am J Hum Genet* 2010;86(1):88-92.
88. Hill A. The environment and disease: association or causation? *J R Soc Med*1965;108(1):32-7.
89. Langer-Gould A, Wu J, Lucas R, et al. Epstein-Barr virus, cytomegalovirus, and multiple sclerosis susceptibility: A multiethnic study. *Neurology* 2017;89(13):1330-37. doi: 10.1212/wnl.0000000000004412 [published Online First: 2017/09/01]
90. Ascherio A, Munger K. EBV and Autoimmunity. *Curr Top Microbiol Immunol* 2015;390(Pt 1):365-85.
91. Casiraghi C, Marquez AC, Shanina I, et al. Latent virus infection upregulates CD40 expression facilitating enhanced autoimmunity in a model of multiple sclerosis. *Scientific reports* 2015;5:13995. doi: 10.1038/srep13995 [published Online First: 2015/09/12]
92. Hauser SL, Waubant E, Arnold DL, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *The New England journal of medicine* 2008;358(7):676-88. doi: 10.1056/NEJMoa0706383
93. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. *Nature reviews Immunology* 2001;1(1):75-82. doi: 10.1038/35095584 [published Online First: 2002/03/22]
94. Ascherio A, Munger KL, Lünemann JD. The initiation and prevention of multiple sclerosis. *Nature reviews Neurology* 2012;8(11):602-12. doi: 10.1038/nrneurol.2012.198
95. Sugden B. Epstein-Barr virus: the path from association to causality for a ubiquitous human pathogen. *PLoS biology* 2014;12(9):e1001939. doi: 10.1371/journal.pbio.1001939 [published Online First: 2014/09/03]

96. Khankhanian P, Cozen W, Himmelstein DS, et al. Meta-analysis of genome-wide association studies reveals genetic overlap between Hodgkin lymphoma and multiple sclerosis. *International journal of epidemiology* 2016;45(3):728-40. doi: 10.1093/ije/dyv364 [published Online First: 2016/03/14]
97. Beasley R, Hwang L, Lin C, et al. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981;2(8256):1129-33.
98. Salahuddin SZ, Ablashi DV, Markham PD, et al. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 1986;234(4776):596-601. [published Online First: 1986/10/31]
99. Schirmer EC, Wyatt LS, Yamanishi K, et al. Differentiation between two distinct classes of viruses now classified as human herpesvirus 6. *Proceedings of the National Academy of Sciences of the United States of America* 1991;88(13):5922-6.
100. Hall CB, Caserta MT, Schnabel KC, et al. Persistence of human herpesvirus 6 according to site and variant: possible greater neurotropism of variant A. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1998;26(1):132-7.
101. Akhyani N, Berti R, Brennan MB, et al. Tissue distribution and variant characterization of human herpesvirus (HHV)-6: increased prevalence of HHV-6A in patients with multiple sclerosis. *The Journal of infectious diseases* 2000;182(5):1321-5. doi: 10.1086/315893
102. Alvarez-Lafuente R, De Las Heras V, Bartolome M, et al. Human herpesvirus 6 and multiple sclerosis: a one-year follow-up study. *Brain pathology (Zurich, Switzerland)* 2006;16(1):20-7.
103. Virtanen JO, Farkkila M, Multanen J, et al. Evidence for human herpesvirus 6 variant A antibodies in multiple sclerosis: diagnostic and therapeutic implications. *Journal of neurovirology* 2007;13(4):347-52. doi: 10.1080/13550280701381332
104. Soldan SS, Berti R, Salem N, et al. Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nature medicine* 1997;3(12):1394-7.
105. Khaki M, Ghazavi A, Ghasami K, et al. Evaluation of viral antibodies in Iranian multiple sclerosis patients. *Neurosciences* 2011;16(3):224-8.
106. Kuusisto H, Hyoty H, Kares S, et al. Human herpes virus 6 and multiple sclerosis: a Finnish twin study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(1):54-8. doi: 10.1177/1352458507080063
107. Xu Y, Linde A, Fredrikson S, et al. HHV-6 A- or B-specific P41 antigens do not reveal virus variant-specific IgG or IgM responses in human serum. *Journal of medical virology* 2002;66(3):394-9.
108. Moore FG, Wolfson C. Human herpes virus 6 and multiple sclerosis. *Acta neurologica Scandinavica* 2002;106(2):63-83.
109. Engdahl E, Gustafsson R, Ramanujam R, et al. HLA-A(*)02, gender and tobacco smoking, but not multiple sclerosis, affects the IgG antibody response against human herpesvirus 6. *Human immunology* 2014;75(6):524-30. doi: 10.1016/j.humimm.2014.03.001 [published Online First: 2014/03/26]
110. Derfuss T, Hohlfeld R, Meinl E. Intrathecal antibody (IgG) production against human herpesvirus type 6 occurs in about 20% of multiple sclerosis patients

and might be linked to a polyspecific B-cell response. *Journal of neurology* 2005;252(8):968-71. doi: 10.1007/s00415-005-0794-z
111. Alenda R, Alvarez-Lafuente R, Costa-Frossard L, et al. Identification of the major HHV-6 antigen recognized by cerebrospinal fluid IgG in multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2014;21(8):1096-101. doi: 10.1111/ene.12435 [published Online First: 2014/04/15]
112. Villoslada P, Juste C, Tintore M, et al. The immune response against herpesvirus is more prominent in the early stages of MS. *Neurology* 2003;60(12):1944-8.
113. Knox KK, Brewer JH, Henry JM, et al. Human herpesvirus 6 and multiple sclerosis: systemic active infections in patients with early disease. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2000;31(4):894-903. doi: 10.1086/318141
114. Voumvourakis KI, Kitsos DK, Tsiodras S, et al. Human herpesvirus 6 infection as a trigger of multiple sclerosis. *Mayo Clinic proceedings* 2010;85(11):1023-30. doi: 10.4065/mcp.2010.0350
115. Ramroodi N, Sanadgol N, Ganjali Z, et al. Monitoring of active human herpes virus 6 infection in Iranian patients with different subtypes of multiple sclerosis. *Journal of pathogens* 2013;2013:194932. doi: 10.1155/2013/194932
116. Mirandola P, Stefan A, Brambilla E, et al. Absence of human herpesvirus 6 and 7 from spinal fluid and serum of multiple sclerosis patients. *Neurology* 1999;53(6):1367-8.
117. Al-Shammari S, Nelson RF, Voevodin A. HHV-6 DNAaemia in patients with multiple sclerosis in Kuwait. *Acta neurologica Scandinavica* 2003;107(2):122-4. [published Online First: 2003/02/13]
118. Berti R, Brennan MB, Soldan SS, et al. Increased detection of serum HHV-6 DNA sequences during multiple sclerosis (MS) exacerbations and correlation with parameters of MS disease progression. *Journal of neurovirology* 2002;8(3):250-6. doi: 10.1080/13550280290049615-1 [published Online First: 2002/06/08]
119. Virtanen JO, Wohler J, Fenton K, et al. Oligoclonal bands in multiple sclerosis reactive against two herpesviruses and association with magnetic resonance imaging findings. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(1):27-34. doi: 10.1177/1352458513490545 [published Online First: 2013/06/01]
120. Ortega-Madueno I, Garcia-Montojo M, Dominguez-Mozo MI, et al. Anti-human herpesvirus 6A/B IgG correlates with relapses and progression in multiple sclerosis. *PloS one* 2014;9(8):e104836. doi: 10.1371/journal.pone.0104836
121. Opsahl ML, Kennedy PG. Early and late HHV-6 gene transcripts in multiple sclerosis lesions and normal appearing white matter. *Brain : a journal of neurology* 2005;128(Pt 3):516-27. doi: 10.1093/brain/awh390
122. Cermelli C, Berti R, Soldan SS, et al. High frequency of human herpesvirus 6 DNA in multiple sclerosis plaques isolated by laser microdissection. *The Journal of infectious diseases* 2003;187(9):1377-87. doi: 10.1086/368166
123. Mameli G, Astone V, Arru G, et al. Brains and peripheral blood mononuclear cells of multiple sclerosis (MS) patients hyperexpress MS-associated retrovirus/HERV-W endogenous retrovirus, but not Human herpesvirus 6. *The*

Journal of general virology 2007;88(Pt 1):264-74. doi: 10.1099/vir.0.81890-0
124. Tejada-Simon MV, Zang YC, Hong J, et al. Cross-reactivity with myelin basic protein and human herpesvirus-6 in multiple sclerosis. *Annals of neurology* 2003;53(2):189-97. doi: 10.1002/ana.10425
125. Cheng W, Ma Y, Gong F, et al. Cross-reactivity of autoreactive T cells with MBP and viral antigens in patients with MS. *Front Biosci (Landmark Ed)* 2012;17:1648-58. [published Online First: 2011/12/29]
126. Dietrich J, Blumberg BM, Roshal M, et al. Infection with an endemic human herpesvirus disrupts critical glial precursor cell properties. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2004;24(20):4875-83. doi: 10.1523/JNEUROSCI.5584-03.2004
127. Leibovitch EC, Jacobson S. Evidence linking HHV-6 with multiple sclerosis: an update. *Current opinion in virology* 2014;9:127-33. doi: 10.1016/j.coviro.2014.09.016 [published Online First: 2014/12/03]
128. Alvarez-Lafuente R, Garcia-Montojo M, De Las Heras V, et al. Herpesviruses and human endogenous retroviral sequences in the cerebrospinal fluid of multiple sclerosis patients. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(5):595-601. doi: 10.1177/1352458507086425
129. Lower R, Lower J, Kurth R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93(11):5177-84.
130. Muir A, Lever A, Moffett A. Expression and functions of human endogenous retroviruses in the placenta: an update. *Placenta* 2004;25 Suppl A:S16-25. doi: 10.1016/j.placenta.2004.01.012
131. Perron H, Geny C, Laurent A, et al. Leptomeningeal cell line from multiple sclerosis with reverse transcriptase activity and viral particles. *Research in virology* 1989;140(6):551-61.
132. Perron H, Garson JA, Bedin F, et al. Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. The Collaborative Research Group on Multiple Sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* 1997;94(14):7583-8.
133. Garson JA, Tuke PW, Giraud P, et al. Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. *Lancet* 1998;351(9095):33.
134. Serra C, Sotgiu S, Mameli G, et al. Multiple sclerosis and multiple sclerosis-associated retrovirus in Sardinia. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* 2001;22(2):171-3.
135. Arru G, Mameli G, Astone V, et al. Multiple Sclerosis and HERV-W/MSRV: A Multicentric Study. *International journal of biomedical science : IJBS* 2007;3(4):292-7.
136. Zawada M, Liwien I, Pernak M, et al. MSRV pol sequence copy number as a potential marker of multiple sclerosis. *Polish journal of pharmacology* 2003;55(5):869-75.
137. Garcia-Montojo M, de la Hera B, Varade J, et al. HERV-W polymorphism in chromosome X is associated with multiple sclerosis risk and with differential

expression of MSRV. *Retrovirology* 2014;11:2. doi: 10.1186/1742-4690-11-2
138. Garcia-Montojo M, Dominguez-Mozo M, Arias-Leal A, et al. The DNA copy number of human endogenous retrovirus-W (MSRV-type) is increased in multiple sclerosis patients and is influenced by gender and disease severity. *PloS one* 2013;8(1):e53623. doi: 10.1371/journal.pone.0053623
139. Laska MJ, Brudek T, Nissen KK, et al. Expression of HERV-Fc1, a human endogenous retrovirus, is increased in patients with active multiple sclerosis. *Journal of virology* 2012;86(7):3713-22. doi: 10.1128/JVI.06723-11
140. Brudek T, Christensen T, Aagaard L, et al. B cells and monocytes from patients with active multiple sclerosis exhibit increased surface expression of both HERV-H Env and HERV-W Env, accompanied by increased seroreactivity. *Retrovirology* 2009;6:104. doi: 10.1186/1742-4690-6-104
141. Nexo BA, Christensen T, Frederiksen J, et al. The etiology of multiple sclerosis: genetic evidence for the involvement of the human endogenous retrovirus HERV-Fc1. *PloS one* 2011;6(2):e16652. doi: 10.1371/journal.pone.0016652
142. Hansen B, Oturai AB, Harbo HF, et al. Genetic association of multiple sclerosis with the marker rs391745 near the endogenous retroviral locus HERV-Fc1: analysis of disease subtypes. *PloS one* 2011;6(10):e26438. doi: 10.1371/journal.pone.0026438
143. de la Hera B, Varade J, Garcia-Montojo M, et al. Human endogenous retrovirus HERV-Fc1 association with multiple sclerosis susceptibility: a meta-analysis. *PloS one* 2014;9(3):e90182. doi: 10.1371/journal.pone.0090182
144. Moyes DL, Goris A, Ban M, et al. HERV-K113 is not associated with multiple sclerosis in a large family-based study. *AIDS research and human retroviruses* 2008;24(3):363-5. doi: 10.1089/aid.2007.0196
145. Tai A, O'Reilly E, Alroy K, et al. Human Endogenous Retrovirus-K18 env as a risk factor in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(9):1175-80. doi: 10.1177/1352458508094641
146. Bianchi I, Lleo A, Gershwin ME, et al. The X chromosome and immune associated genes. *Journal of autoimmunity* 2012;38(2-3):J187-92. doi: 10.1016/j.jaut.2011.11.012
147. Sotgiu S, Arru G, Mameli G, et al. Multiple sclerosis-associated retrovirus in early multiple sclerosis: a six-year follow-up of a Sardinian cohort. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2006;12(6):698-703.
148. Antony JM, Izad M, Bar-Or A, et al. Quantitative analysis of human endogenous retrovirus-W env in neuroinflammatory diseases. *AIDS research and human retroviruses* 2006;22(12):1253-9. doi: 10.1089/aid.2006.22.1253
149. Perron H, Germi R, Bernard C, et al. Human endogenous retrovirus type W envelope expression in blood and brain cells provides new insights into multiple sclerosis disease. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(12):1721-36. doi: 10.1177/1352458512441381
150. Perron H, Lazarini F, Ruprecht K, et al. Human endogenous retrovirus (HERV)-W ENV and GAG proteins: physiological expression in human brain and pathophysiological modulation in multiple sclerosis lesions. *Journal of neurovirology* 2005;11(1):23-33. doi: 10.1080/13550280590901741
151. Schmitt K, Richter C, Backes C, et al. Comprehensive analysis of human endogenous retrovirus group HERV-W locus transcription in multiple

sclerosis brain lesions by high-throughput amplicon sequencing. *Journal of virology* 2013;87(24):13837-52. doi: 10.1128/JVI.02388-13
152. Saresella M, Rolland A, Marventano I, et al. Multiple sclerosis-associated retroviral agent (MSRV)-stimulated cytokine production in patients with relapsing-remitting multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2009;15(4):443-7. doi: 10.1177/1352458508100840
153. Perron H, Jouvin-Marche E, Michel M, et al. Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response in vitro, by inducing polyclonal Vbeta16 T-lymphocyte activation. *Virology* 2001;287(2):321-32. doi: 10.1006/viro.2001.1045
154. Firouzi R, Rolland A, Michel M, et al. Multiple sclerosis-associated retrovirus particles cause T lymphocyte-dependent death with brain hemorrhage in humanized SCID mice model. *Journal of neurovirology* 2003;9(1):79-93. doi: 10.1080/13550280390173328
155. Kremer D, Schichel T, Forster M, et al. Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Annals of neurology* 2013;74(5):721-32. doi: 10.1002/ana.23970 [published Online First: 2013/07/10]
156. Madeira A, Burgelin I, Perron H, et al. MSRV envelope protein is a potent, endogenous and pathogenic agonist of human toll-like receptor 4: Relevance of GNbAC1 in multiple sclerosis treatment. *Journal of neuroimmunology* 2016;291:29-38. doi: 10.1016/j.jneuroim.2015.12.006 [published Online First: 2016/02/10]
157. Antony JM, van Marle G, Opii W, et al. Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nature neuroscience* 2004;7(10):1088-95. doi: 10.1038/nn1319
158. Antony JM, Ellestad KK, Hammond R, et al. The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes. *Journal of immunology (Baltimore, Md : 1950)* 2007;179(2):1210-24.
159. Sutkowski N, Conrad B, Thorley-Lawson DA, et al. Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. *Immunity* 2001;15(4):579-89.
160. Sutkowski N, Chen G, Calderon G, et al. Epstein-Barr virus latent membrane protein LMP-2A is sufficient for transactivation of the human endogenous retrovirus HERV-K18 superantigen. *Journal of virology* 2004;78(14):7852-60. doi: 10.1128/JVI.78.14.7852-7860.2004
161. Hsiao FC, Tai AK, Deglon A, et al. EBV LMP-2A employs a novel mechanism to transactivate the HERV-K18 superantigen through its ITAM. *Virology* 2009;385(1):261-6. doi: 10.1016/j.virol.2008.11.025 [published Online First: 2008/12/17]
162. Lycke J, Svennerholm B, Hjelmquist E, et al. Acyclovir treatment of relapsing-remitting multiple sclerosis. A randomized, placebo-controlled, double-blind study. *Journal of neurology* 1996;243(3):214-24.
163. Bech E, Lycke J, Gadeberg P, et al. A randomized, double-blind, placebo-

controlled MRI study of anti-herpes virus therapy in MS. *Neurology* 2002;58(1):31-6.
164. Rice GP, Hartung HP, Calabresi PA. Anti-alpha4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology* 2005;64(8):1336-42. doi: 10.1212/01.WNL.0000158329.30470.D0
165. Selter RC, Biberacher V, Grummel V, et al. Natalizumab treatment decreases serum IgM and IgG levels in multiple sclerosis patients. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(11):1454-61. doi: 10.1177/1352458513477229
166. Sokal EM, Hoppenbrouwers K, Vandermeulen C, et al. Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an Epstein-Barr virus vaccine in healthy young adults. *The Journal of infectious diseases* 2007;196(12):1749-53. doi: 10.1086/523813
167. Derfuss T, Curtin F, Guebelin C, et al. A phase IIa randomised clinical study of GNbAC1, a humanised monoclonal antibody against the envelope protein of multiple sclerosis-associated endogenous retrovirus in multiple sclerosis patients. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014(s) doi: 10.1177/1352458514554052
168. Kremer D, Forster M, Schichel T, et al. The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514560926

3.11 publication in chapter 3

Tao C, Simpson Jr S, Taylor BV, van der Mei I. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. Journal of the neurological sciences. 2017;372:239-249.

Section 3.11 has been removed for copyright or proprietary reasons.

Chapter 4 Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis

4.1 Preface

In this chapter, we address aim 1 of the thesis - the examination of the association between latitude and age of symptom onset and to what extent ultraviolet radiation levels may underlie such an association. This chapter has been published in journal of neurology, neursurgery& psychiatry, year 2016, volume 87, page 1343-1349. The manuscript presented in this chapter has been published. The typeset version of the manuscript as it appeared in the journal is in Appendix. The text of this chapter is the same as the published version.

4.2 Abstract

Background: Age at onset (AAO) in multiple sclerosis (MS) is an important marker of disease severity and may have prognostic significance. Understanding what factors can influence AAO may shed light on the aetiology of this complex disease, and have applications in the diagnostic process.

Methods: The study cohort of 22,162 eligible patients from 21 countries was extracted from the MSBase Registry. Only MS patients aged ≥16 years were included. To reduce heterogeneity, only centres of largely European descent were included for analysis. AAO was defined as the year of the first symptom suggestive of inflammatory central nervous system demyelination. Predictors of AAO were evaluated by linear regression.

Results: Compared with those living in lower latitudes (19.0-39.9 °), onset of symptoms was 1.9 years earlier for those at higher latitudes (50.0-56.0 °) ($p=3.83x10^{-23}$). A reciprocal relationship was seen for ambient UVR (ultraviolet radiation), with a significantly increasing age at onset for MS patients per each quartile increment of ambient UVR ($p=1.56x10^{-17}$). We found that the AAO of female patients was approximately 5 months earlier than male patients (p=0.002). AAO of progressive-onset MS patients were approximately 9 years later than relapsing-onset patients ($p=1.40x10^{-265}$).

Conclusion: An earlier AAO in higher latitude regions was found in this worldwide European-descent cohort and correlated inversely with variation in latitudinal UVR. These results suggest that environmental factors which act at the population level may significantly influence disease severity characteristics in genetically susceptible populations.

4.3 Introduction

Multiple sclerosis (MS) is a complex autoimmune disorder of the central nervous system characterised by inflammatory demyelination on a background of progressive neurodegeneration. The aetiology of MS is still uncertain[1]; however, a complex interplay between genetic and environmental factors likely contributes to disease onset. Of the environmental risk factors linked to MS, the robust latitudinal gradients of prevalence[2] and incidence rate[3] have been among the most consistent and striking findings in MS epidemiology[4]. Indeed, we previously showed a positive association between latitude and MS prevalence with a 1.04 change in prevalence per degree-latitude ($p<0.001$)[2], while Alonso & Hernán[3] demonstrated that MS incidence

increased 30% in women and 50% in men per each ten-degree increment of latitude. The explanations of this geographic variation have largely been considered to reflect changes in ambient UVR (ultraviolet radiation) and/or concomitant variations in vitamin D sufficiency[5] although this is by no means the only potential explanation[6 7].

Using space-time cluster analysis, studies have demonstrated a high degree of clustering of residence was associated with a lower age at onset (AAO) of MS[8 9]. From these studies, it is reasonable to assume that there may exist an association between latitude and AAO for MS patients. However, little data is available on whether a higher latitude or lower ambient UVR is associated with a lower AAO.

The latitudinal gradient has been shown to be present largely in populations of European descent[2] and also to vary by MS phenotype[10]. It is unclear whether the latitudinal gradient of MS also affects clinical characteristics, although some recent studies[11 12] have demonstrated a significant relationship between latitude and positivity for cerebrospinal fluid (CSF) oligoclonal bands (OCBs). A recent study in Japan has also confirmed this relationship between latitude and abnormal CSF findings, with a significant association of positive CSF findings (OR=4.08) and OCB positivity (OR=2.57) when comparing northern to southern Japan[13] in 180 MS patients diagnosed with the 2010 McDonald criteria[14].

The association between AAO and the prognosis of MS patients is complicated. A prospective population-based cohort in southeast Wales[15] indicated that complete recovery from the onset event varied in different age groups, with full recovery more common (87.4%) in the youngest group (less than 18 years old), compared to only

68% in the oldest (greater than 44 years old). Another cohort study also found that an older AAO could accelerate the accumulation of disability in MS[16], and this association was independent of disease duration and early relapse frequencies. However, despite the fact that early onset MS patients manifest a comparatively moderate clinical course, the younger onset was associated with a younger attainment of disability milestones[17]. While these studies have been consistent in their determination that AAO is a determinant of the progression of MS, less is known about the factors that influence the age of symptom onset in MS, with studies focusing on genetic, viral, metabolic and hormonal factors showing variable results[18-20].

In the present study, we sought to evaluate whether there was any evidence of a latitudinal variation in the age of symptom onset of MS and if so, whether such variation could be ascribed to a similar UV-based mechanism or if other factors were at play.

4.4 Methods

4.4.1 The MSBase Registry

The MSBase Registry is an international collaboration that has established a prospective longitudinal dataset of MS patients' neurological records from a number of sites worldwide. Presently, the Registry has 59 centres in 26 countries in Europe, North America, South America, Asia Minor, South Asia and Australia. Participating centres enter data in or near real-time using a secure internet-based database.

The MSBase Registry was approved by the Melbourne Health Human Research Ethics Committee, and local ethics committees in all participating centres gave regional approval for participation in the MSBase Registry. Written informed consent was obtained from all participants who provided data to the Registry.

4.4.2 Criteria for data extraction

All patients fulfilled either the 2005 or 2010 McDonald criteria[14 21] for diagnosis of MS, with the data being extracted and edited on 31st May 2014, a total of 30,415 participants were included (Figure 1).

4.4.3 Participant inclusion criteria

Our dataset was restricted to patients with clinically diagnosed MS as per the McDonald criteria 2010[14] and to adult-onset MS (excluding patients with AAO less than 16 years old[22 23]).The Atlas of MS[24] has demonstrated that the prevalence for MS between Asian and European populations varies significantly, from 1/100,000 to >180/100,000. In the present sample, other characteristics like the populations mean age and the sex ratio of the source populations also differed significantly between sites. For example, sex ratio of source population (female/male) was 0.94 in non-European-descent centres (Isfahan, Iran; Kuwait City, Kuwait; Mumbai, India) while it was 1.06 in other centres, a significant difference ($p=2.74x10^{-6}$). Likewise, the mean age of the source population differed significantly, with mean age of 33.39 years in non-European-descent centres compared to 39.33 years in European-descent centres ($p=8.34x10^{-6}$). This marked variation in population characteristics and the heterogeneity of main effects between the two centre subsets led to our excluding

these three centres from our analysis (Figure 1).These exclusion left a total of 22,162 patients from 52 centres in 21 countries.

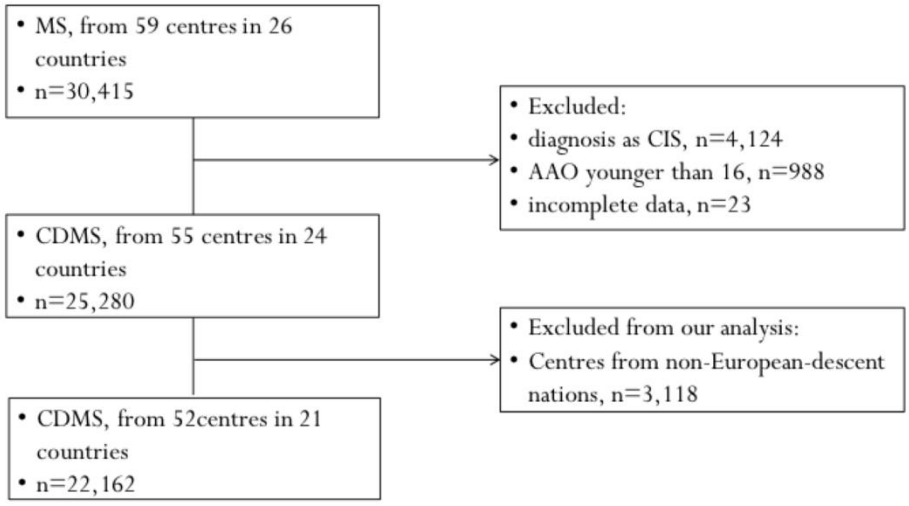

Figure 4.1 Flow chart showing inclusion criteria of the sample for the present analysis. CDMS: clinically diagnosed MS; CIS: clinically isolated syndrome.

4.4.4 Factors of interest

Participant birthdate, sex, first recorded MS type (relapsing-onset, progressive-onset) and date of symptom onset, and current MS types, as well as the location of the registered MSBase centre (city, country) were recorded for every participant.

Age at onset

Age at onset was defined as the age of the first validated symptom(s) consistent with multiple sclerosis.

Latitude

The latitude of each study centre was derived from Google maps (https://www.google.com.au/maps/). For our purposes, latitudes were recoded to all be positive, though we retained information on the hemisphere of the study centre to allow for evaluation of differences by hemisphere. For a better presentation of the latitudinal gradient and according to the sample distribution in our study, latitude was categorised into three strata: $<40^{o}$, $40\text{-}50^{o}$, and $>50^{o}$. Using other cut points such a s quartiles or 10 degree increments did not significantly alter the data.

Solar radiation values

We created a UVB variable for each MSBase participant based on the latitude and longitude of each treatment centre. UVB (wavelengths 280-320nm) was our focus in this study, since only shorter-wavelength UVB radiation can induce the cutaneous synthesis of vitamin D. UV data were collected from the Solar Radiation Database (SoDa) service. Hourly values for clear sky were calculated and summed to daily or monthly values. UVB intensity was defined as radiation received per area, expressed as Wh/m^2 (watt hour per square meter). Mean monthly values from December to March were used to calculate the winter mean UVB for the Northern Hemisphere and from June to August for the Southern Hemisphere. Research[25] has demonstrated that UVR can modulate the immune response independent of the effect of vitamin D, however all UV measurements are highly correlated e.g. erythemally weighted UVR and UVB ($r=0.99$, $p<0.001$) therefore there was no difference in outcomes when using any measure of UVR.

4.4.5 Potential confounding variables

Population mean age

It was reasonable to assume that a higher mean age in the source population could impact on the number of cases available for study, given the average age of prevalent cases being in early-mid adulthood. In terms of our dataset, we found a positive association between population mean age and AAO ($r=0.06$, $p<0.001$) and latitude ($p=0.49$, $r<0.001$), therefore population average age was included in the multivariable model. Low variance inflate factor allows the inclusion of both variables in the multivariable model. We obtained the mean age of the source population in each treatment centre from the most recent national demographic statistics available for each country.

Sex ratio in each treatment centre

Since female patients exhibit an earlier AAO, and there was a negative association between sex ratio (female/male) and latitude ($r=-0.29$, $p<0.001$), this was added to the multivariable model. From the sex distribution of MS patients in each treatment centre we calculated the sex ratio (female/males).

MS type ratio in each treatment centre

AAO in relapsing-onset patients is typically earlier than progressive-onset patients, and we found a positive association between the ratio of MS type (progressive-onset/relapsing-onset) at each centre and latitude ($r=0.38$, $p<0.001$), therefore MS type ratio in each treatment centre was added to the multivariable model.

4.4.6 Statistical analysis

This study was analysed with cox proportional analysis and linear analysis initially, which showing a similar direction and statistical significance. For an easier

interpretation and clinical application (beta coefficient), linear regression was used to assess potential predictors of AAO. Since we excluded all patients less than 16 years old, the distribution of AAO in our dataset was left-skewed. Log-transformation was applied to reduce heteroskedasticity and thus satisfy the requirements of linear regression. All coefficients were back-transformed to present on the original scale at the mean of model covariates, however, to facilitate interpretation of results. Due to the significant heterogeneity of the sample size in each treatment centre, all analyses were weighted by the square root of the sample size in each treatment centre.

To assess the seasonality of the AAO, we fitted a sinusoidal model to data on AAO and the month (t) when the patient was born: $AAO=\beta_0+\beta_1\sin(2\pi t/12)+\beta_2\cos(2\pi t/12)$. The ANOVA F-test was used to decide whether there was significant seasonal variation.

All statistical analyses were undertaken using Stata/SE 12.1 (College Park, TX).

4.5 Results

4.5.1 Participant characteristics

Our analysis dataset included a total of 22,162 patients from 52 centres in 21 countries (Table 1). In keeping with known MS distributions, most patients were female (70.4%) and of relapsing-remitting course (91.5%). The distribution of the cohort was such that most patients were from the Northern Hemisphere (81.4%), particularly Europe (67.2%), while participants from Australia also contributed a large proportion (15.7%). The mean AAO in this cohort was 32.3 years. Supplemental Table 1 shows the characteristics of the total cohort, overall and by centre.

Table 4.1 List of countries included in our primary analysis

	Number of patients (%)	Recruiting centres	Mean latitude of study centres	Mean winter UVB (Wh/m^2)
Mexico	69 (0.31)	1	19.4 °N	14.3
Cuba	134 (0.60)	1	23.1 °N	11.8
Israel	83 (0.37)	1	31.9 °N	6.3
Argentina	643 (2.90)	2	33.0 °S	6.5
Australia	3,470 (15.66)	8	35.0 °S	5.7
Malta	87 (0.39)	1	35.9 °N	5.0
Spain	2,571 (11.60)	6	40.6 °N	3.8
United States	80 (0.36)	1	40.7 °N	3.6
Turkey	1,166 (5.26)	2	41.1 °N	3.2
Portugal	254 (1.15)	1	41.2 °N	3.9
Macedonia	23 (0.10)	1	42.0 °N	3.2
Italy	5,090 (22.97)	9	43.0 °N	2.9
Romania	17 (0.08)	1	44.4 °N	2.7
Canada	2,866 (12.93)	2	46.1 °N	2.0
Hungary	96 (0.43)	4	47.7 °N	1.6
France	80 (0.36)	1	48.9 °N	2.2
Czech Republic	1,787 (8.06)	1	50.1 °N	1.4
Belgium	417 (1.88)	1	50.9 °N	1.3
Netherlands	2,568 (11.59)	5	51.6 °N	1.2
Northern Ireland	236 (1.06)	2	54.5 °N	1.0
Denmark	425 (1.92)	1	55.7 °N	0.8
Total	22,162 (100)	52		

Wh/m2=watt per square meter; Mean latitude: average of latitudes of centres in the same country.
Mean winter UVB: Mean monthly values from December to March were used to calculate the winter mean UVB for northern hemisphere and from June to August were used for southern hemisphere.
Values of mean latitude of study centres and mean winter UVB described the average values of latitude and winter UVB in each country, the values here would not be used in the statistical analysis.

4.5.2 Factors associated with AAO

Latitude and UVB

Our primary factor of interest for AAO was study centre latitude. In the univariable analysis, latitude as a continuous linear factor was significantly negatively associated with AAO (p=0.01), every 10-degree increment of latitude associated with a 0.3-year earlier onset. Evaluating this as a categorical term in Table 2, we found a clear dose-

dependent response. On adjustment for relevant covariates, this association became much more dose-dependent, with those of the highest latitude stratum having nearly 1.9-years earlier age at onset than those of the lowest (Figure 2). When we evaluated latitude as a continuous variable in the multivariable analysis, a 10^{o} increase in latitude was associated with a 0.82-year earlier onset ($p<0.001$). We did not include UV in our multivariable analysis, due to the significant collinearity between the level of UV and latitude. Restricting to relapsing-onset patients, every 10-degree increment of latitude was associated with a 0.76-year earlier onset ($p<0.001$). In supplementary Table 2, we show the stepwise process of the adjustment used in our multivariable model.

The results for the association between AAO and winter UVB were similar to the latitudinal ones, and the results were predictably of high correlation ($r=-0.96$, $p<0.001$) between latitude and UVB. In the univariable analysis, no strong dose-response relationship was observed, but the association and dose-dependency were enhanced after adjustment for confounders. A clear dose-dependent association ($p<0.001$) appeared with those of the lowest UVB level having nearly 2 years earlier AAO than those of the highest UVB category in the multivariable analysis (Figure 3). Using summer UVB and winter erythemally weighted UV, the positive relationship between higher sun exposure and later AAO persisted ($p<0.001$).

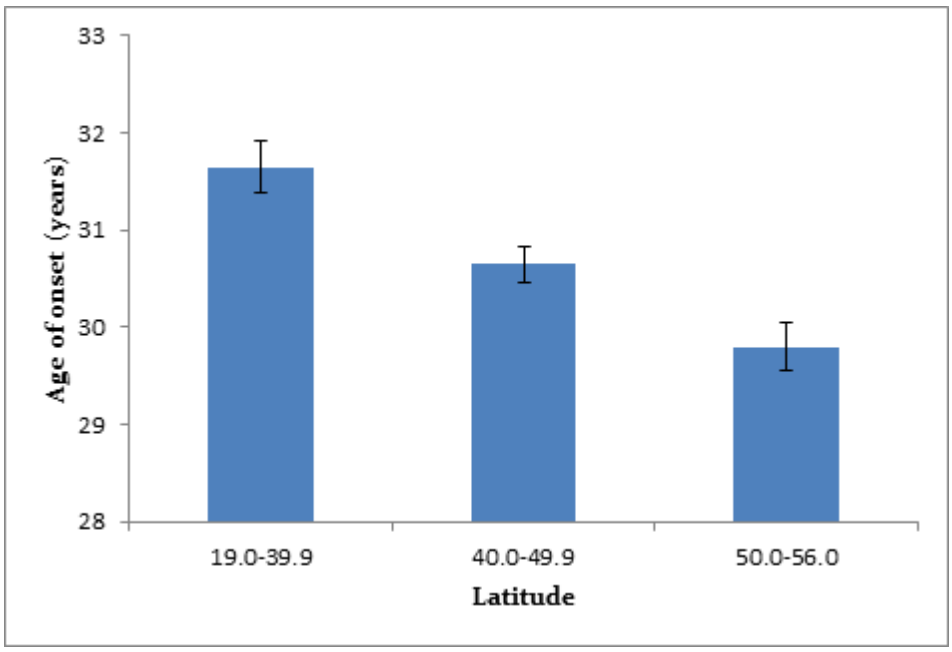

Figure 4.2Adjusted age at onset in each latitude category.

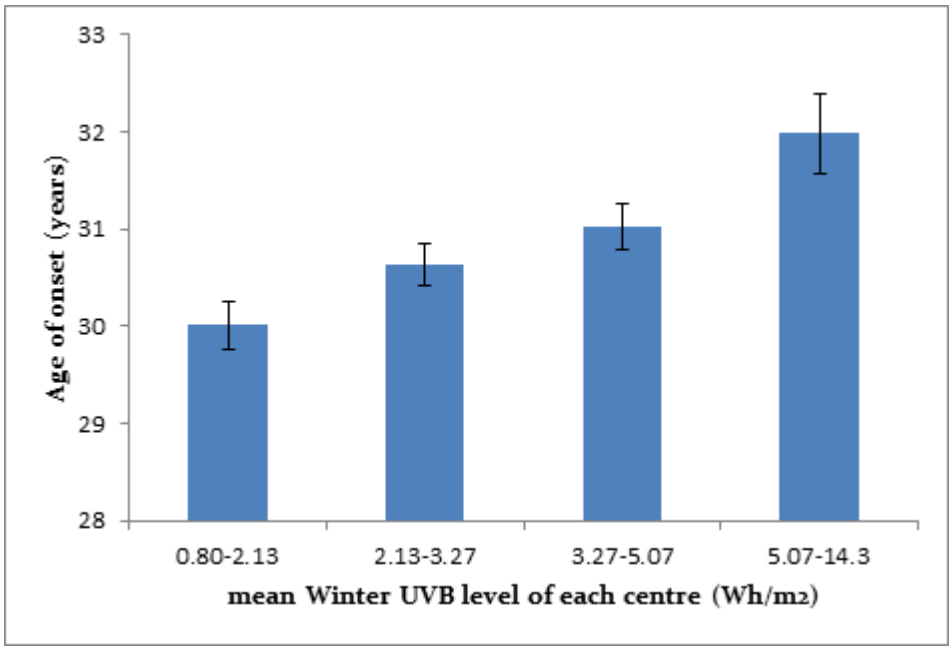

Figure 4.3Adjusted age at onset in each winter ultraviolet B category.

Sex

Female patients' AAO were on average 0.88 years (95% CI: 0.60-1.17; p<0.001) younger than male patients in our univariable analysis. After adjustment for other relevant variables, this magnitude reduced to 0.43 years (95% CI: 0.15-0.70; p=0.002). Restricting to relapsing-onset patients, female patients still showed 0.58 years earlier onset than male patients (p<0.001). The latitudinal gradient of AAO was not significantly different between males and females (p=0.10 for interaction).

MS type

Progressive-onset patients exhibited a much later age at onset than relapsing-onset MS patients (β: 9.29; 95% CI: 8.74-9.84; p<0.001) (Table 2). This association was quite robust to adjustment (β: 8.90; 95% CI: 8.35-9.45; p<0.001). The latitudinal gradient of AAO was not significantly different between relapsing-onset and progressive-onset cases (p=0.36 for interaction). Subgroup analysis of the association between latitude and ASO were significant in both relapsing-onset (p<0.001) and progressive-onset (p=0.002) patients.

Season of Birth

Birth dates were relatively evenly distributed in the four seasons. We explored season of birth (Figure 4) and its association with age at onset using a sinusoidal model, finding no seasonal pattern (F=0.66, p=0.52).

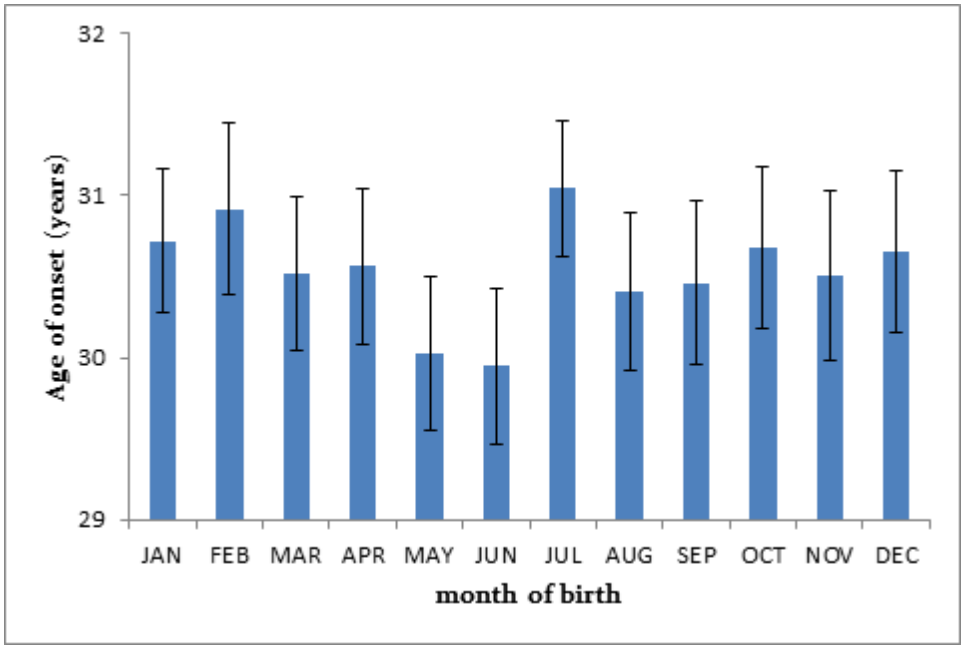

Figure 4.4Mean age at symptom onset by month of birth. All southern hemisphere birth dates were moved 6 months forward to sync with northern hemisphere seasons.

Table 4.2 Univariable and multivariable analysis of factors associated with MS age at onset

	Univariable			Multivariable	
	N (%)	β (95% CI)	P	β (95% CI)	P
Degree of latitude [a]					
19.0-39.9	5,850 (26.40)	31.47 (31.21,31.72)		31.65 (31.40, 31.91)	
40.0-49.9	10,879 (49.09)	***-0.88 (-1.19,-0.56)***	***<0.001***	***-1.01 (-1.32, -0.69)***	***<0.001***
50.0-56.0	5,433 (24.51)	***-1.22 (-1.58,-0.86)***	***<0.001***	***-1.86 (-2.22, -1.49)***	***<0.001***
Trend			***<0.001***		***<0.001***
Winter UV (Wh/m^2) [b]					
5.07-14.3	2,626 (11.85)	31.97 (31.59,32.36)		31.98 (31.57, 32.38)	
3.27-5.07	6,429 (29.01)	***-1.53 (-1.98,-1.08)***	***<0.001***	***-0.95 (-1.44, -0.46)***	***<0.001***
2.13-3.27	6,865 (30.98)	***-1.10 (-1.55,-0.65)***	***<0.001***	***-1.35 (-1.81, -0.89)***	***<0.001***
0.8-2.13	6,242 (28.17)	***-1.50 (-1.96,-1.05)***	***<0.001***	***-1.96 (-2.44, -1.49)***	***<0.001***
Trend			***0.001***		***<0.001***
Sex [a]					
Male	6,550 (29.56)	31.35 (31.11,31.59)		31.00 (30.77, 31.23)	
Female	15,612 (70.44)	***-0.88 (-1.17,-0.60)***	***<0.001***	***-0.43 (-0.70, -0.15)***	***0.002***
MS type [a]					
Relapsing-onset	20,277 (91.49)	30.02 (29.89,30.15)		30.03 (29.90, 30.16)	
Progressive-onset	1,885 (8.51)	***+9.29 (8.74,9.84)***	***<0.001***	***+8.90 (8.35, 9.45)***	***<0.001***
Season of birth [a]					
Spring	5,483 (24.74)	30.51(30.25,30.77)		30.51(30.26, 30.75)	
Summer	6,131 (27.66)	+0.24 (-0.12, 0.60)	0.18	+0.20(-0.14, 0.55)	*0.25*
Autumn	5,025 (22.67)	+0.25 (-0.13, 0.62)	0.19	+0.21(-0.15, 0.57)	*0.24*
Winter	5,523 (24.92)	+0.37 (0.00, 0.73)	*0.05*	+0.36(0.00,0.71)	*0.05*

Relapsing-onset: relapsing-remitting MS and secondary progressive MS;
Progressive-onset: primary progressive MS and progressive relapsing MS

[a]Each model with the factor of interest was adjusted for sex, latitude, MS type, average population age of each centre, sex ratio of each centre and MS type ratio of each centre; b: adjusted for sex, MS type, sex ratio of each centre and MS type ratio of each centre.

4.6 Discussion

Using one of the largest cohorts to date with 22,162 patients from 21 countries, we evaluated the role of latitude and age of MS symptom onset in adult, European-descent patients. We found evidence that those living at a higher latitude had a significantly younger AAO, with every 10 $^{\circ}$ increase in latitude associated with around a ten month earlier AAO. The results were consistent when latitude was analysed as a categorical variable, demonstrating a strong inverse association between latitude and AAO, such that those in the highest latitudinal range had a nearly 2-years earlier onset than those in the lowest latitudinal range. Accordingly, we found a positive association between a lower winter ambient UV level and an earlier age at onset, these results neatly in harmony due to the known relationship between UV levels and latitude. Sex and MS type showed expected differences in AAO; however, we found no difference in AAO by season/month of birth.

In line with our results, one previous study[26] also found that latitude was negatively associated with AAO in RRMS patients, although this association was not significant (p=0.10). However, the magnitude of the latitudinal association observed in that study (1.3 years change in per 10 ^{o}latitude) is greater than the findings in our study, the absence of significance simply reflecting their smaller sample size of 987 patients. The difference in those findings to the present work likely reflects the difference in study samples (mostly Caucasian US military veterans vs. a worldwide collaborative patient records database).

Variation of ambient winter UV is a possible but not the only explanation for this latitudinal variation in AAO, in line with previous research indicating associations between vitamin D/UV in MS onset and progression[27 28]. We utilised mean winter UV intensity in different centres as a predicted variable to analyse the association with AAO. Due to the close relationship between latitude and UVB radiation ($r=-0.96$, $p<0.001$ in our study), we were unable to extricate the individual effects to determine which was the more potent driver of effect. This likely reflects the less sensitive mode of assessing latitude or UV, by utilising surrogate latitude or ambient UV levels at the study centre rather than assessing individual participant exposure. However, the similarity of the two models also suggested UV radiation is the main factor underlying the association of latitude and onset age. Notably, other factors such as socioeconomicstatus and EBV infection may also contribute to the latitudinal gradient of ASO.

A recent study in Japan demonstrated that latitude had a more significant impact on prevalence of MS than UVB[29], and some studies have proposed that factors other than UVR may lead to the latitudinal gradient[7]. The latitudinal gradient of Epstein-Barr virus (EBV) infection prevalence may also partly explain the earlier onset at high latitudinal regions. A recent meta-analysis demonstrated that an increased rate of EBV infection in higher latitudinal areas occurred in both MS patients and the general populations[6]. Another study[30] found a similar geographical distribution between infectious mononucleosis and MS that may also contribute to the latitudinal gradient in prevalence and also potentially age at onset. However, an ecological study[31] performed in Australia showed a closer relationship between ambient ultraviolet

radiation and MS prevalence (r=-0.91, p=0.01) when compared with the relationship of latitudes (r=0.89, p=0.02). Another study conducted by our group including 136 MS patients found that increasing exposure to sun in adolescence was not associated with AAO[32]. However, this study found that skin phenotype was associated with AAO, which may be driven by the differential ability of skin types to absorb UV and synthesise vitamin D. A recent study[33] with a larger sample size (n=540) suggested that cumulative sun exposure (more than 16 weeks) in fall/winter in the 9 to 15 years age group was associated with 2.1 years later AAO (p=0.02), persisting on adjustment for sunscreen use, cod liver oil intake, sex and skin type. Genetic variation is another potential factor that may account for the latitudinal gradient of AAO, our previous study[2] demonstrated enhancement of the association between latitude and prevalence of MS by genotype particularly *HLA-DRB*1, another study[34] showed that *HLA-DRB1* genotype could account for up to 52% of the latitudinal gradient of prevalence

We also showed that women exhibit an earlier AAO than men and this relationship has been supported by many other studies. One study[15] with 1,424 patients conducted in southeast Wales showed a significant difference of AAO between sexes (male 31.2, female 29.3, p=0.002). This difference is in the same direction as our results, but the magnitude was larger than ours. Likely the difference in magnitude reflects the different populations assessed. It may be that latitudinal or sex effects may be appreciably greater or lesser in local regions, but in a multicentre database-based study like ours, such regional differences may average out to yield a reduced magnitude of difference. Earlier puberty in females may be a candidate explanation for the earlier onset in females, as a study has found a positive association between

age of menarche and AAO[35]. Progressive-onset patients showed approximately 9 years later onset than relapsing-onset patients. Some research has also shown a positive relationship between additional year of AAO and progressive-onset phenotype[36]. The study[36] within the Lyon Multiple sclerosis Cohort showed a similar magnitude to our study with their mean age at onset in PPMS and RRMS being 40.6 and 29.4-years, respectively.

Studies in a number of countries with a high prevalence of MS have demonstrated that more people with MS were born in spring and relatively few in autumn[37]. An international study[38] including 11,415 patients from 15 countries has shown that this seasonal association existed but did not correlate with variation in UVR exposure ($p=0.086$). A multicentre longitudinal study[39] conducted in Australia also demonstrated a higher risk of MS for those born in November-December when compared to those born in May-June ($p<0.01$). Our study showed that there was no clear association with month or season of birth and AAO. This is in keeping with the finding that the association between MS risk and month of birth was debatable when enough variables were included in the analysis[40] (especially those variables associated with regional and temporal variation).

We made the assumption that patients generally were born and lived in the same latitude where the study centres were located. We found no evidence to indicated that MS patients were more likely to move to other areas after disease onset. This is not an unreasonable expectation; however, our inability to exclude patients who fell outside this assumption may affect our results. However, we believe that this misclassification

error should be evenly distributed and would not impact the association of latitude and AAO.

In MSBase study, cases were diagnosed with Poser, Polman or McDonald's criteria, which may lead to some difference in the diagnosis of MS. We have adjusted for the ratio of MS type and sex to account for the different mixture of patients in each study centre. Moreover, the primary aim of the study was to analyse the association between latitude and age at onset, and no evidence of the significantly differential diagnosis by latitude was seen. We therefore believed that the potential spectrum effect would not influence the results materially.

Our analysis had remarkable statistical power provided by 22,162 patients from an international clinic-based and private practice-based study. Accordingly, while we have noted statistical significance, we were appropriately focused on magnitudes of difference in AAO. Our study does have some limitations, however. Firstly, we had no individual information on diet, dietary supplementation (particularly with vitamin D), and likewise individual genetic characteristics was not available. The lack of personal information leads to a lower explainable variance in our study and insufficient control of the confounding bias. Secondly, despite the similarity in demographic data between MSBase and other observational studies, our sample could be somewhat skewed because it included patients from clinics and private practices from developed countries with a universal health cover, and excluded those who did not attend those clinics (e.g. severely disabled). It is difficult to predict how that would have affected the association between latitude and ASO. In order to control for some selection bias, all joined MS Base centres agreed to database all available

patients[41]. We also weighted by the count of patients in each centre in our study, which decreased such inter-centre variation. However, we did not have data about the total number of MS patients in each centre or the proportion of patients enrolled in MSBase study in each centre. It would be hard to predict whether any sampling error would be differential by age of onset. As a result, it is not possible to predict whether it affected our main associations of interest and in which direction. Another limitation in this study was the potential measurement error of ASO. ASO of some cases could be inaccurate especially when the onset was progressive, which could have reduced our associations towards the null. However, no evidence was seen that the potential measurement error of ASO was differential in different latitudinal region. Moreover, when restricting to relapsing-onset cases, the association between latitude and ASO was still significant. Finally, birth cohort effect could influence the association between latitude and ASO materially especially when different generations of cases were included in multi-centres with unknown period of recruitment. However, the sensitivity analysis according to the date of birth (1920-1940, 1941-1950, 1951-1960, 1961-1970, 1971-1980, 1981-1997) all showed a significant association between increased latitude and decreased ASO. Therefore, it was reasonable to consider that birth cohort effect would not influence the result materially.

In conclusion, this study confirms the presence of a significant inverse latitudinal gradient in the onset age in multiple sclerosis; such that each 10 ^{o}increased latitude was associated with a 0.8 years earlier onset. Our results also supported that variation of UVR exposure may play a role in the latitudinal gradient, although other factors like differential timing of EBV infection and genetic background may also have a

role. No difference in onset age by season or month of birth was seen, however, suggesting that while such in-utero effects may modulate MS risk, the onset age may be determined by latitudinally varying exposures occurring after birth. The increased likelihood of patients living in high latitude areas to suffer an earlier onset of MS may lead to improved understanding of MS aetiopathogenesis.

4.7 Postscript

This chapter demonstrated the latitudinal gradient of age at onset in patients with MS, and UVR is the possible candidate underlying this phenomenon. The next chapter investigates the role of a range of individual variables on age of symptom onset in patients with first demyelinating event.

4.8 References

1. Mechelli R, Annibali V, Ristori G, et al. Multiple sclerosis etiology: beyond genes and environment. *Expert review of clinical immunology* 2010;6(3):481-90. doi: 10.1586/eci.10.11
2. Simpson Jr. S, Blizzard L, Otahal P, et al. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *Journal of Neurology, Neurosurgery and Psychiatry* 2011;82(10):1132-41. doi: doi:10.1136/jnnp.2011.240432
3. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. *Neurology* 2008;71(2):129-35. doi: 10.1212/01.wnl.0000316802.35974.34
4. Kurtzke JF. General features on the prevalence of multiple sclerosis. *J Indian Med Prof* 1964;11:4896-901.
5. Handel AE, Giovannoni G, Ebers GC, et al. Environmental factors and their timing in adult-onset multiple sclerosis. *Nature reviews Neurology* 2010;6(3):156-66. doi: 10.1038/nrneurol.2010.1 [published Online First: 2010/02/17]
6. Disanto G, Pakpoor J, Morahan JM, et al. Epstein-Barr virus, latitude and multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(3):362-5. doi: 10.1177/1352458512451942
7. Sajedi SA, Abdollahi F. Geomagnetic disturbances may be environmental risk factor for multiple sclerosis: an ecological study of 111 locations in 24 countries. *BMC neurology* 2012;12:100. doi: 10.1186/1471-2377-12-100

[published Online First: 2012/09/25]
8. Riise T, Klauber MR. Relationship between the degree of individual space-time clustering and age at onset of disease among multiple sclerosis patients. *International journal of epidemiology* 1992;21(3):528-32.
9. Riise T, Gronning M, Klauber MR, et al. Clustering of residence of multiple sclerosis patients at age 13 to 20 years in Hordaland, Norway. *American journal of epidemiology* 1991;133(9):932-9.
10. Taylor BV, Pearson JF, Clarke G, et al. MS prevalence in New Zealand, an ethnically and latitudinally diverse country. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(12):1422-31. doi: 10.1177/1352458510379614
11. Dobson R, Ramagopalan S, Davis A, et al. Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *Journal of neurology, neurosurgery, and psychiatry* 2013;84(8):909-14. doi: 10.1136/jnnp-2012-304695
12. Lechner-Scott J, Spencer B, de Malmanche T, et al. The frequency of CSF oligoclonal banding in multiple sclerosis increases with latitude. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(7):974-82. doi: 10.1177/1352458511431729
13. Niino M, Sato S, Fukazawa T, et al. Latitude and HLA-DRB1 alleles independently affect the emergence of cerebrospinal fluid IgG abnormality in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2015 doi: 10.1177/1352458514560924
14. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Annals of neurology* 2011;69(2):292-302. doi: 10.1002/ana.22366
15. Cossburn M, Ingram G, Hirst C, et al. Age at onset as a determinant of presenting phenotype and initial relapse recovery in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(1):45-54. doi: 10.1177/1352458511417479
16. Scalfari A, Neuhaus A, Daumer M, et al. Age and disability accumulation in multiple sclerosis. *Neurology* 2011;77(13):1246-52. doi: 10.1212/WNL.0b013e318230a17d
17. Confavreux C, Vukusic S. Age at disability milestones in multiple sclerosis. *Brain : a journal of neurology* 2006;129(Pt 3):595-605. doi: 10.1093/brain/awh714
18. Harbo HF, Isobe N, Berg-Hansen P, et al. Oligoclonal bands and age at onset correlate with genetic risk score in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(6):660-8. doi: 10.1177/1352458513506503
19. Sorosina M, Esposito F, Guaschino C, et al. Inverse correlation of genetic risk score with age at onset in bout-onset and progressive-onset multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514561910
20. Bove R, Chitnis T. The role of gender and sex hormones in determining the onset and outcome of multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458513519181 [published

Online First: 2014/02/25]
21. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Annals of neurology* 2005;58(6):840-6. doi: 10.1002/ana.20703 [published Online First: 2005/11/12]
22. Renoux C, Vukusic S, Confavreux C. The natural history of multiple sclerosis with childhood onset. *Clinical neurology and neurosurgery* 2008;110(9):897-904. doi: 10.1016/j.clineuro.2008.04.009
23. Compston A et al. McAlpine's multiple sclerosis. [Churchill Livingstone] 2006.
24. Atlas of MS 2013: Mapping Multiple Sclerosis Around the World. London: Multiple Sclerosis International Federation; 2013. http://www.msif.org/about-ms/publications-and-resources/ (Accessed 10 Oct 2014).
25. Lucas RM, Ponsonby AL, Dear K, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011;76(6):540-8. doi: 10.1212/WNL.0b013e31820af93d
26. McDowell TY, Amr S, Langenberg P, et al. Time of birth, residential solar radiation and age at onset of multiple sclerosis. *Neuroepidemiology* 2010;34(4):238-44. doi: 10.1159/000297749 [published Online First: 2010/03/20]
27. Ebers GC. Environmental factors and multiple sclerosis. *Lancet neurology* 2008;7(3):268-77. doi: 10.1016/S1474-4422(08)70042-5
28. Simpson S, Taylor B, Blizzard L, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS. *Annals of neurology* 2010:n/a-n/a. doi: 10.1002/ana.22043
29. Kinoshita M, Obata K, Tanaka M. Latitude has more significant impact on prevalence of multiple sclerosis than ultraviolet level or sunshine duration in Japanese population. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* 2015 doi: 10.1007/s10072-015-2150-0
30. Ascherio A, Munger KL. Epstein-barr virus infection and multiple sclerosis: a review. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 2010;5(3):271-7. doi: 10.1007/s11481-010-9201-3 [published Online First: 2010/04/07]
31. van der Mei IA, Ponsonby AL, Blizzard L, et al. Regional variation in multiple sclerosis prevalence in Australia and its association with ambient ultraviolet radiation. *Neuroepidemiology* 2001;20(3):168-74. doi: 54783
32. van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *Bmj* 2003;327(7410):316. doi: 10.1136/bmj.327.7410.316
33. McDowell TY, Amr S, Culpepper WJ, et al. Sun exposure, vitamin D and age at disease onset in relapsing multiple sclerosis. *Neuroepidemiology* 2011;36(1):39-45. doi: 10.1159/000322512
34. Handel AE, Handunnetthi L, Giovannoni G, et al. Genetic and environmental factors and the distribution of multiple sclerosis in Europe. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2010;17(9):1210-4. doi: 10.1111/j.1468-1331.2010.03003.x
35. Sloka JS, Pryse-Phillips WE, Stefanelli M. The relation between menarche and the

age of first symptoms in a multiple sclerosis cohort. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2006;12(3):333-9. [published Online First: 2006/06/13]
36. Confavreux C, Vukusic S. Natural history of multiple sclerosis: a unifying concept. *Brain : a journal of neurology* 2006;129(Pt 3):606-16. doi: 10.1093/brain/awl007
37. Willer CJ, Dyment DA, Sadovnick AD, et al. Timing of birth and risk of multiple sclerosis: population based study. *Bmj* 2005;330(7483):120. doi: 10.1136/bmj.38301.686030.63
38. Verheul F, Smolders J, Trojano M, et al. Fluctuations of MS births and UV-light exposure. *Acta neurologica Scandinavica* 2013;127(5):301-8. doi: 10.1111/ane.12007 [published Online First: 2012/09/14]
39. Staples J, Ponsonby AL, Lim L. Low maternal exposure to ultraviolet radiation in pregnancy, month of birth, and risk of multiple sclerosis in offspring: longitudinal analysis. *Bmj* 2010;340:c1640. doi: 10.1136/bmj.c1640
40. Fiddes B, Wason J, Kemppinen A, et al. Confounding underlies the apparent month of birth effect in multiple sclerosis. *Annals of neurology* 2013;73(6):714-20. doi: 10.1002/ana.23925
41. Butzkueven H, Chapman J, Cristiano E, et al. MSBase: an international, online registry and platform for collaborative outcomes research in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2006;12(6):769-74. [published Online First: 2007/02/01]

4.9 Supplementary Tables

Table 4.3 List of centres

	Number of patients (%)	Mean age of onset	Mean age of source population	Mean latitude
Centres				
Huixquilucan	69 (0.27)	31.31	28.90	19.41 ºN
Havana	134 (0.53)	33.67	39.89	23.05 ºN
Brisbane	162 (0.64)	32.49	36.25	27.47 ºS
Cordoba	17 (0.07)	32.44	34.72	31.40 ºS
Be'er Ya'akov	83 (0.33)	31.39	31.33	31.94 ºN
Perth	71 (0.28)	33.73	36.84	31.95 ºS
Newcastle	462 (1.83)	33.84	38.64	32.93 ºS
Sydney	745 (2.95)	34.10	37.21	33.87 ºS
Buenos Aires	626 (2.48)	32.37	34.43	34.60 ºS
Adelaide	257 (1.02)	34.31	39.00	34.93 ºS
Msida	87 (0.34)	32.40	41.50	35.90 ºN
Sevilla	216 (0.85)	31.73	39.12	37.39 ºN
Macarena	1,408 (5.57)	31.99	39.12	37.67 ºN
Melbourne	1,415 (5.6)	33.27	37.28	37.81 ºS
Geelong	98 (0.39)	33.11	39.55	38.15 ºS
Madrid	516 (2.04)	32.21	40.06	40.42 ºN
Salerno	445 (1.76)	31.61	41.92	40.68 ºN
New York	80 (0.32)	34.46	36.39	40.71 ºN
Avellino	246 (0.97)	33.03	42.96	40.91 ºN

Trabzon	646 (2.56)	30.59	29.05	41.00 ºN
Bari	1,370 (5.42)	29.76	42.08	41.12 ºN
Porto	254 (1.00)	32.27	40.05	41.16 ºN
Samsun	520 (2.06)	29.67	28.69	41.29 ºN
Barcelona	294 (1.16)	33.19	41.34	41.39 ºN
Skopje	23 (0.09)	31.84	35.48	42.00 ºN
Chieti	1,412 (5.59)	32.08	44.61	42.35 ºN
Hobart	260 (1.03)	35.14	39.10	42.88 ºS
Vizcaya	114 (0.45)	34.70	44.22	43.22 ºN
Macerata	379 (1.50)	29.89	44.71	43.30 ºN
San Sebastian	23 (0.09)	35.62	43.05	43.32 ºN
Florence	245 (0.97)	33.77	45.54	43.77 ºN
Bucharest	17 (0.07)	29.07	38.09	44.43 ºN
Modena	400 (1.58)	33.75	43.70	44.65 ºN
Parma	207 (0.82)	33.79	44.81	44.80 ºN
Pavia	386 (1.53)	29.62	45.23	45.18 ºN
Montreal	2,153 (8.52)	33.04	38.75	45.51 ºN
Levis	713 (2.82)	34.11	37.57	46.74 ºN
Budapest	68 (0.27)	28.41	42.04	47.50 ºN
Debrecen	14 (0.06)	29.31	39.58	47.53 ºN
Gyor	10 (0.04)	25.53	40.60	47.69 ºN
Nyiregyhaza	4 (0.02)	25.80	38.74	47.95 ºN
Paris	80 (0.32)	30.84	38.48	48.86 ºN
Prague	1,787 (7.07)	29.22	40.00	50.08 ºN
Brussels	417 (1.65)	31.38	37.80	50.85 ºN
Sittard	598 (2.37)	33.98	40.62	51.00 ºN
Roosendaal	136 (0.54)	36.68	40.62	51.54 ºN
Den Bosch	382 (1.51)	34.48	40.62	51.70 ºN
Nijmegen	1,181 (4.67)	33.97	40.62	51.81 ºN
Gouda	271 (1.07)	35.94	40.62	52.01 ºN
Craigavon	193 (0.76)	33.27	35.98	54.45 ºN
Belfast	43 (0.17)	32.48	35.98	54.60 ºN
Copenhagen	425 (1.68)	32.65	35.42	55.68 ºN
Total	**22,162 (100)**			

Table 4.4 Stepwise analysis

	β (95% CI)	P	Adjustments & rationales
Latitude[a]			In this univariable analysis, we observed a dose-dependent association between latitude and AAO.
19.0-39.9	31.47 (31.21,31.72)		
40.0-49.9	-0.88 (-1.19,-0.56)	$4.09x10^{-8}$	
50.0-56.0	-1.22 (-1.58,-0.86)	$4.01x10^{-11}$	
Trend		$3.68x10^{-11}$	
Latitude[b]			In this model, we adjusted for sex for each individual. Since female patients exhibited an earlier AAO, and the negative association between the count of female patients and latitude (r=-0.02, p=0.001), this adjustment was reasonable. However, the adjustment for sex did change the magnitude of latitude materially;
19.0-39.9	31.46 (31.21, 31.72)		
40.0-49.9	-0.87 (-1.19, -0.56)	$5.12x10^{-8}$	

50.0-56.0	-1.22 (-1.58, -0.86)	$4.25x10^{-11}$	
Trend		$3.91x10^{-11}$	
Latitude[c]			In this model, we adjusted for sex and MS type for each individual. Since PPMS patients exhibit a later AAO, and the positive association between the count of PPMS patients and latitude (r=0.09, p<0.001), this adjustment was reasonable. Moreover, adjustment for MS type changed the association of latitude and AAO materially.
19.0-39.9	31.50 (31.25, 31.74)		
40.0-49.9	-0.86 (-1.16, -0.55)	$3.75x10^{-8}$	
50.0-56.0	-1.41 (-1.76, -1.06)	$2.49x10^{-15}$	
Trend		$2.32x10^{-15}$	
Latitude[d]			In this model, we adjusted for sex and MS type for each individual and the average age of population in each centre. Since a positive association between population mean age and AAO (r=0.06, p<0.001) and latitude (p=0.49, r<0.001), it was reasonable to adjust for population average age.
19.0-39.9	31.52 (31.26, 31.77)		
40.0-49.9	-0.89 (-1.21, -0.57)	$4.18x10^{-8}$	
50.0-56.0	-1.44 (-1.79, -1.08)	$2.76x10^{-15}$	
Trend		$4.20x10^{-15}$	
Latitude[e]			In this model, we adjusted for sex and MS type for each individual and the average age of population and sex ratio (F/M) of MS patients in each centre. Since female patients exhibit an earlier AAO, and the negative association between the ratio of female patients and latitude (r=-0.29, p<0.001), this adjustment was reasonable.
19.0-39.9	31.58 (31.33, 31.84)		
40.0-49.9	-1.03 (-1.35, -0.72)	$1.21x10^{-10}$	
50.0-56.0	-1.46 (-1.82, -1.11)	$9.38x10^{-16}$	
Trend		$2.14x10^{-15}$	
Latitude[f]			In this model, we adjusted for sex and MS type for each individual and the average age of population, sex ratio (F/M) of MS patients and MS type ratio (POMS/ROMS) in each centre. Since RRMS patients exhibit an earlier AAO, and the positive association between the ratio of MS type and latitude (r=0.49, p<0.001), this adjustment was reasonable. The adjustment for the ratio of MS type enhanced the association between latitude and AAO, we could observe a clear dose-dependent association now. The proportion of PPMS patients were much higher in European nations than non-European nations (p<0.001), so this MS type ratio could modulate the magnitude and direction materially.
19.0-39.9	31.65 (31.40, 31.91)		
40.0-49.9	-1.01 (-1.32, -0.69)	$3.91x10^{-10}$	
50.0-56.0	-1.86 (-2.22, -1.49)	$3.83x10^{-23}$	
Trend		$3.78x10^{-23}$	

POMS: progressive onset MS; ROMS: relapsing-onset MS; AAO: age at onset

4.10 Publication in chapter 4

Tao C, Simpson S, Jr., van der Mei I, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. Journal of neurology, neurosurgery, and psychiatry. 2016;87(12):1343-1349.

Section 4.10 has been removed for copyright or proprietary reasons.

Chapter 5 Tobacco smoking, progressive-onset, cerebral dysfunction are associated with a delayed onset of multiple sclerosis and marijuana use with an earlier onset

5.1 Preface

The previous chapter investigated the associations between surrogate latitude and age at onset. This chapter will address aim 2 of the thesis - the effects of knowing/emerging risk factors on age of symptom onset using the AusImmune Study.

5.2 Abstract

Background: Age at symptom onset (ASO) is a prognostic factor that could affect disability development in patients with multiple sclerosis (MS). A number of factors are now known to influence the risk of MS, but their influence on the ASO is less well-investigated.

Objective: Examine the associations between known or emerging MS risk factors and ASO.

Methods: Multicentre study; cases (n=279) aged 18–59 years with a first clinical diagnosis of central nervous system demyelination, recruited at four Australian centres (latitudes 27 ℃S to 43 ℃S), from 1 November 2003 to 31 December 2006. Environmental/behavioural and initial symptoms variables were recorded at baseline interview through questionnaire and neurological review. Linear regression was used to assess the association between risk factors and ASO.

Results: A history of tobacco smoking was associated with 3.05-years later ASO (p=0.002); a history of marijuana use was associated with 6.03-years earlier ASO (p<0.001); progressive-onset cases had 5.61-years later ASO (p=0.001); an initial presentation of bowel & bladder and cerebral functional impairment were associated with 3.39 (p=0.017) and 4.37-years (p=0.006) later ASO, respectively. Other factors,

including sex, offspring number, latitude of study site, history of infectious mononucleosis, *HLA-DR15 &HLA-A2* genotype, 25(OH)D levels, and ultraviolet radiation exposure were not significantly associated with ASO.

Conclusion: We found a novel association between a history of tobacco smoking and later onset, whereas marijuana use was associated with earlier onset. Behavioural factors might be important drivers of MS onset timing and may aid in our understanding of MS pathogenesis.

5.3 Introduction

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (CNS)[1]. *HLA-DR15*01* genotype[2], lower vitamin D[3] or exposure to ultraviolet radiation (UVR)[4], tobacco use[5], and evidence of past infection with Epstein-Barr virus[6] are the main factors implicated in MS risk. Our group recently demonstrated that these risk factors could explain 63.8% of the attributable risk[7], with 53.3% of that solely due to the environmental factors. Other factors such as *HLA-A2* genotype[8] (protective), offspring number[9] (protective), and marijuana use[5] (detrimental), have some evidence of involvement in MS risk.

While a number of studies have examined risk factors for the onset of MS, far fewer have tested associations with age at symptom onset (ASO), which is the age at which first symptoms suggestive of future MS occur. Some studies showed that lower sun exposure in adolescence was associated with earlier ASO[10 11]. One cohort study (n=895 cases) found no difference in ASO between never and ever smokers (32.29 and 32.75 years, respectively)[12]. One study (n=816) found that cases with *HLA-DR15* risk genotype had roughly 2.5-years earlier onset[13], a later pooled analysis (n=2,201) showed no association between *HLA-DR15* genotype and ASO.[14] The association between *HLA-A2* genotype and ASO has also yielded inconsistent findings: Smestad and colleagues found a weak correlation between *HLA-A2* genotype and a later ASO ($p=0.07$)[15], but another study showed no association[16]. Regarding the onset type of

MS, our recent large study[17] has found that primary-progressive MS (PPMS) patients had approximately 9 years later onset of initial symptoms than RRMS patients ($p<0.001$).

In view of the conflicting evidence presented above, this paper examines the association between risk factors for MS onset and ASO in a cohort recruited soon after the first diagnosis of CNS demyelination (FCD).

5.4 Methods

The Ausimmune Study was an Australian multicentre case-control study[18]. Participants were aged 18-59 years and resident in a study region: Brisbane city (latitude 27° South), Newcastle city and surrounds (33°S), Geelong city and the Western Districts of Victoria (37°S), or the state of Tasmania (approximately 41-43°S). Incident cases (n=282) were referred to the study by medical specialists, following a first clinical diagnosis of CNS demyelination. A study neurologist confirmed the date and symptomatology of the demyelinating event(s) that led to study participation and conducted a full neurologic examination. In subsequent follow-up (AusLong study)[19], three cases were found to have a non-MS condition, and 19 cases were confirmed as PPMS. Among all 279 cases, 219 had been diagnosed as MS at 5-year review. Among 260 relapsing-onset cases, 219 had their first ever symptom of CNS demyelination (FDE) during the study recruitment period (1 Nov 2003 to 31 Dec 2006), with remaining cases having had a prior, previously unrecognised neurological event.

The Ausimmune Study was approved by nine regional Human Research Ethics Committees. All participants gave written informed consent.

5.4.1 Measurements

Demographic, environmental, behavioural and neurological data were collected by self-reported questionnaire and face-to-face interview. Serum samples and biometric

measures were taken at face-to-face interview. Mean interval between study entry and date of FDE was 1.37 years.

Clinical presentation was divided into seven categories (pyramid, cerebellar, brainstem, sensory, bowel & bladder, cerebral and visual) and confirmed by a neurologist via face-to-face interview at baseline. With in-person interview at study entry, questionnaire data collected included: a detailed smoking history of tobacco and marijuana use (ever smoked, current smoking status, age started/stopped smoking); history of infectious mononucleosis ("have you ever had glandular fever"; those who did not know whether they had a history of IM were coded as not having had IM); number and age of all live births; and for females only, the age at menarche. Participants completed a life calendar from age 6 years noting, for each year of life, the location of residence, and leisure time in the sun in summer and winter. With the latitude and longitude of the location, average daily ambient erythemally weighted UVR was calculated for every year of life for each participant[4]. For each participant, UVR dose (kJ/m^2) was summed in relevant periods of life by multiplication of the average hours per day in each season outside and average UVR for that season. Annual UVR exposure was assigned by adding the summer and winter UVR dose.

Serum concentrations of 25(OH)D were measured using liquid chromatography dual mass spectrometry at study completion. Skin reflectance on the buttock (non-sun-exposed site) was measured using a hand-held spectrophotometer (Minolta CM-2500D) to estimate cutaneous melanin density[4]. The genotyping of *HLA-DR15* (SNP rs9271366) was performed by the SNPline method (KBiosciences, HoddesdonHerts, UK). The genotyping of *HLA-A2* (SNP rs2844821 on Illumina Custom MS Chip) was performed by the Hussman Institute for Human Genomics, University of Miami.

5.4.2 Data analysis

ASO was calculated by subtracting the date of birth from the date of first symptom onset (as determined by review of data by the study neurologist team). Where only the year of symptom onset was recorded, the onset date was assigned as 15th of June of that year and if only the month was known it was assigned as the 15th of the month

(month of FDE was not sure in 27 cases, but results were robust when these cases were dropped (data not shown)).

Because 25(OH)D levels vary substantially by season, we deseasonalised 25(OH)D levels in each study centre using a sinusoidal function, as described previously[4]. The 25(OH)D level was modelled as both a continuous and categorical variable (using commonly used cut-points, <50, 50-75, 76-100, >100 nmol/L). Measurement of body mass index was after study entry, so we did not adjust for it in the multivariable model.

Logbinomial regression was used to assess the association between two categorical variables. Linear regression was used to assess the association between potential predictors and ASO. We mostly examined risk factors that occurred prior to the initial symptom onset to ensure proper temporality. 25(OH)D was measured at the baseline review. For variables that were inherently associated with age (e.g. smoking, sun exposure, offspring number), we summarized the information prior to a specific age and examined the associations with ASO after this age to obtain a less biased estimate. This ensured that the participants included in the analysis had an equal opportunity of exposure, but it substantially reduced the power (analysis restricting to those with a later ASO). It also assumes that there is nothing unusual about those with a later ASO. Where participants with an early onset were excluded from the study, the distribution was left skewed. Box-Cox power transformation was then applied to reduce heteroskedasticity and thus satisfy the requirements of linear regression. All coefficients presented in the results were back-transformed on the original scale of age.

In preliminary analyses, participants in Tasmania had a later ASO than other study centres (mean difference between Tasmania and other centres: 3.19 years, 95% CI 0.73-5.65, p=0.011). The higher ASO is likely to have been due to the higher average age of the Tasmanian population; however, due to collinearity between the population mean age and study centre (r=0.91, p<0.001), adjustment for population mean age was not possible. We therefore divided cases into five-year ASO groups (15-19 years,

20-24 years, 25-29 years, 30-34 years, 35-39 years, 40-44 years, 45-49 years, 50-54 years, 55-59 years) in each study centre, and standardised the ASO to the age distribution in the whole Australian population. After standardisation, ASO (from highest to lowest latitude: 37.2, 37.6, 37.0 & 37.2 years, respectively) was similar in the four different study centres.

In the basic multivariable model for each exposure of interest, we adjusted for sex and study centre, and MS onset type (relapsing vs progressive onset). For sun exposure analysis, we did not adjust for study centre due to collinearity (latitude and ambient UVR are strongly negatively correlated). We adjusted for buttock melanin density to take natural skin type into consideration. For smoking analyses, we further adjusted for tobacco or marijuana smoking, due to the significant positive correlation between them ($r=0.40$, $p<0.001$).

After the univariable and multivariable analyses of each category of exposures, we built a mutually adjusted model including all significant exposures, and examined the total variance explained by these exposures. The relative contribution of each variable was calculated as the change of the residual sum of squares (SSs) when comparing the model without the variable with the full model.

To examine whether our key findings were robust, we conducted a number of sensitivity analyses. First, a number of cases had their FDE before the recruitment period and the reliability of the symptom onset date was somewhat uncertain. Therefore, we conducted a sensitivity analysis restricted to cases with FDE during the recruitment period (all these patients were relapsing-onset). Second, as a small percentage of our cases may never convert to MS, we performed a separate analysis restricted to cases who had converted to MS by a 5-year follow-up[19]. Third, we were concerned that some associations were the result of an age effect or a cohort effect. To examine this we used the controls (n=545)and assigned the same ASO of the cases to their age and sex-matched controls. If association were also observed in controls, then that is likely due to an age or cohort effect.

All statistical analyses were undertaken using Stata/SE 12.1 (College Park, TX).

5.5 Results

The mean ASO for the total cohort was 37.3 years and 76.7% were females. ASO for those who had converted to MS at 5-year follow-up (n=217) was 37.0 years and for cases with FDE during the recruitment period (n=219) was 36.9 years.

5.5.1 Sex, HLA-DR15, HLA-A2 and history of infectious mononucleosis

We found no significant associations between ASO and sex, history of IM, *HLA-DR15* genotype, or *HLA-A2* genotype (Table 1).

5.5.2 Vitamin D and UVR dose

We explored season of FDE and its association with AAO, finding no seasonal pattern (F=0.61, p=0.72). There was no association between deseasonalised 25(OH)D and ASO, either as a continuous (p=0.88) or categorical term (p=0.67) (Table 1). Time between FDE and 25(OH)D level being taken did not modify the association between 25(OH)D and ASO (test for interaction p=0.38). Cumulative UVR dose (summer, winter, and combined) within 15 years prior to onset was not associated with ASO (Table 1). Different risk windows (e.g. 5 and 10 years) or UVR dose during adolescence (6-15 years) also showed no convincing associations with subsequent ASO (Supplementary Table 2).

Table 5.1 Associations between sex, IM history, HLA-DR15 genotype, HLA-A2 genotype, 25(OH)D, UV exposure and age of symptom onset

		Univariable		Multivariable	
	No. (%)	**β (95% CI)**	**p**	**β (95% CI)**	**p**
Sex [a]					
Male	65 (23.30)	37.50 (35.17, 39.83) [c]		37.21 (34.90, 39.52) [c]	
Female	214 (76.70)	-0.28 (-2.94, 2.38)	0.842	+0.15 (-2.49, 2.80)	0.913
Past history of IM [a]					
No	200 (72.99)	37.61 (36.30, 38.92) [c]		37.7 (36.41, 39.00) [c]	
Yes	74 (27.01)	-1.18 (-3.70, 1.34)	0.355	-1.33 (-3.83, 1.18)	0.302
HLA-DR15 (rs9271366) [a]					

AA	106 (43.80)	37.19 (35.38, 39.00) [c]		36.89 (34.91, 38.86) [c]	
AG	123 (50.83)	+0.40 (-2.07, 2.86)	0.748	+1.42 (-1.26, 4.09)	0.303
GG	13 (5.37)	+3.18 (-2.26, 8.63)	0.254	+4.74 (-2.43, 11.91)	0.203
Trend			0.380		0.151
HLA-A2 (rs2844821) [a]					
AA	117 (54.42)	36.93 (35.22, 38.64) [c]		37.17 (35.43, 38.92) [c]	
AG	98 (45.58)	+1.79 (-0.74, 4.31)	0.173	+1.38 (-1.24, 4.01)	0.298
Season of FDE [b]					
Spring	70 (25.09)	36.9 (34.47, 39.34) [c]		37.2 (34.69, 39.7) [c]	
Summer	72 (25.81)	+1.00 (-2.27, 4.26)	0.548	0.01 (-3.39, 3.42)	0.989
Autumn	61 (21.86)	+0.05 (-3.28, 3.37)	0.978	-0.42 (-3.8, 2.97)	0.813
Winter	76 (27.24)	+0.40 (-2.87, 3.66)	0.813	0.43 (-2.89, 3.76)	0.804
Deseasonalised 25(OH)D, continuous (per 10 nmol/L increase) [b]		-0.04 (-0.48, 0.40)	0.872	-0.03 (-0.48, 0.41)	0.880
Deseasonalised 25(OH)D [b]					
< 50 nmol/L	33 (15.57)	36.95 (33.68, 40.22) [c]		36.92 (33.64, 40.19) [c]	
50 - 75 nmol/L	70 (33.02)	-0.19 (-4.16, 3.77)	0.923	-0.33 (-4.32, 3.65)	0.869
76 - 100 nmol/L	70 (33.02)	-0.68 (-4.65, 3.28)	0.741	-0.75 (-4.72, 3.22)	0.713
> 100 nmol/L	39 (18.40)	+1.26 (-3.18, 5.71)	0.579	+1.19 (-3.25, 5.64)	0.602
Trend			0.667		0.673
Winter UV dose in the 15 years before onset [b]					
0 - 0.93 x10^2 kJ/m^2	64 (24.81)	39.62 (37.43, 41.81) [c]		39.68 (37.47, 41.9) [c]	
> 0.93 - 1.59x10^2 kJ/m^2	65 (25.19)	-3.05 (-6.12, 0.02)	0.053	***-3.37 (-6.46, -0.28)***	***0.032***
> 1.59 - 2.51x10^2 kJ/m^2	64 (24.81)	-1.95 (-5.04, 1.14)	0.219	-2.06 (-5.22, 1.09)	0.203
>2.51 - 8.47x10^2 kJ/m^2	65 (25.19)	-1.43 (-4.51, 1.65)	0.358	-1.37 (-4.50, 1.76)	0.389
Trend			0.530		0.568
Summer UV dose in the 15 years before onset [b]					
0 - 5.53 x10^2 kJ/m^2	64 (24.71)	39.82 (37.66, 41.97) [c]		39.54 (37.35, 41.73) [c]	
> 5.53 - 7.37x10^2 kJ/m^2	65 (25.10)	***-3.91 (-6.91, -0.91)***	***0.011***	***-3.75 (-6.77, -0.73)***	***0.015***
> 7.37 - 9.99x10^2 kJ/m^2	65 (25.10)	***-3.95 (-6.95, -0.95)***	***0.010***	***-3.87 (-6.93, -0.81)***	***0.013***
>9.99 - 18.99x10^2 kJ/m^2	65 (25.10)	+0.38 (-2.66, 3.42)	0.810	+1.07 (-2.15, 4.28)	0.523
Trend			0.812		0.649
Annual UV dose in the 15 years before onset [b]					
0 - 6.87 x10^2 kJ/m^2	64 (24.81)	39.35 (37.18, 41.52) [c]		39.09 (36.89, 41.3) [c]	
> 6.87 - 9.30x10^2 kJ/m^2	65 (25.19)	***-3.13 (-6.15, -0.10)***	***0.043***	-3.01 (-6.07, 0.04)	0.053
> 9.30 - 12.00x10^2 kJ/m^2	64 (24.81)	***-3.42 (-6.45, -0.38)***	***0.027***	***-3.26 (-6.37, -0.15)***	***0.040***
>12.00 - 22.46x10^2 kJ/m^2	65 (25.19)	+0.85 (-2.21, 3.91)	0.587	+1.41 (-1.79, 4.61)	0.386
Trend			0.643		0.482

Statistical significance ($p<0.05$) was denoted in bold and italics.
a: adjusted for sex, MS onset type and study centre; b: adjusted for sex, MS onset type and buttock melanin density; c: mean ASO of the reference group

Abbreviations: MS, multiple sclerosis; IM, infectious mononucleosis; UV, ultraviolet; FDE, first demyelinating event.

5.5.3 Onset type and initial symptoms

We also explored the association between initial symptoms and ASO, and it was acknowledged that these initial symptoms with their preceding pathological process

occurred more or less simultaneously with ASO. However, we believed that these analyses may provide a valuable contribution the mechanisms underlying MS onset.

ASO of progressive-onset patients was 5.61-years later than relapsing-onset patients (p=0.013) (Table 2). There was no association between the occurrence of pyramidal, brainstem, sensory, visual dysfunction and ASO (Table 2). Cases with bowel/bladder and cerebral function impairment had 3.49-years (p=0.017) and 4.37-years (p=0.006) later onset compared to those without these symptoms, respectively, persisting on restriction to relapsing-onset cases.

Table 5.2Associations between onset type/initial symptoms and age of symptom onset

		Univariable analysis		Multivariable analysis	
	No. (%)	**β (95% CI)**	**p**	**β (95% CI)**	**p**
MS onset type					
Relapsing-onset	258 (92.47)	36.99 (35.84, 38.15) [b]		36.95 (35.8, 38.09) [b]	
Progressive-onset	21 (7.53)	***+4.86 (0.44, 9.28)***	***0.031***	***+5.61 (1.17, 10.05)***	***0.013***
Onset symptoms					
Pyramid function-no	150 (58.37)	37.51 (35.98, 39.04) [b]		37.86 (36.33, 39.38) [b]	
Pyramid function-yes	107 (41.63)	+0.21 (-2.17, 2.58)	0.861	-0.52 (-2.94, 1.90)	0.672
Cerebellar function-no	185 (72.27)	36.89 (35.53, 38.26) [b]		37.01 (35.66, 38.37) [b]	
Cerebellar function-yes	71 (27.73)	***+2.83 (0.24, 5.41)***	***0.032***	+2.52 (-0.08, 5.12)	0.062
Brainstem function-no	192 (75.59)	37.43 (36.06, 38.79) [b]		37.4 (36.06, 38.75) [b]	
Brainstem function-yes	62 (24.41)	-0.13 (-2.90, 2.64)	0.931	0.15 (-2.59, 2.89)	0.920
Sensory function-no	114 (45.6)	37.6 (35.83, 39.37) [b]		37.56 (35.81, 39.3) [b]	
Sensory function-yes	136 (54.4)	-0.48 (-2.88, 1.92)	0.703	-0.33 (-2.70, 2.05)	0.791
Bowel & Bladder function-no	210 (79.55)	36.84 (35.56, 38.12) [b]		36.85 (35.58, 38.12) [b]	
Bowel & Bladder function-yes	54 (20.45)	***+3.39 (0.57, 6.20)***	***0.019***	***+3.49 (0.63, 6.34)***	***0.017***
Cerebral function-no	218 (83.52)	36.71 (35.47, 37.95) [b]		36.87 (35.64, 38.1) [b]	
Cerebral function-yes	43 (16.48)	***+5.12 (2.08, 8.15)***	***0.001***	***+4.37 (1.28, 7.46)***	***0.006***
Visual function-no	178 (70.92)	37.64 (36.25, 39.03) [b]		37.74 (36.38, 39.10) [b]	
Visual function-yes	73 (29.08)	-0.80 (-3.38, 1.77)	0.539	-1.00 (-3.54, 1.54)	0.439
Onset symptoms in relapsing-onset cases					
Pyramid function-no	147 (61.51)	37.22 (35.70, 38.75) [b]		37.35 (35.84, 38.86) [b]	
Pyramid function-yes	92 (38.49)	-0.09 (-2.55, 2.37)	0.942	-0.36 (-2.80, 2.09)	0.773
Cerebellar function-no	179 (74.27)	36.55 (35.18, 37.92) [b]		36.55 (35.19, 37.91) [b]	
Cerebellar function-yes	62 (25.73)	***+2.82 (0.11, 5.52)***	***0.041***	***+2.91 (0.21, 5.60)***	***0.034***
Brainstem function-no	179 (74.9)	36.79 (35.39, 38.18) [b]		36.78 (35.41, 38.16) [b]	
Brainstem function-yes	60 (25.1)	+1.06 (-1.72, 3.83)	0.452	+1.14 (-1.61, 3.90)	0.418
Sensory function-no	106 (45.3)	37.15 (35.34, 38.96) [b]		37.12 (35.33, 38.91) [b]	
Sensory function-yes	128 (54.7)	-0.29 (-2.73, 2.15)	0.809	-0.21 (-2.63, 2.21)	0.866
Bowel & Bladder function-no	201 (81.71)	36.52 (35.23, 37.81) [b]		36.43 (35.16, 37.71) [b]	
Bowel & Bladder function-yes	45 (18.29)	***+3.33 (0.31, 6.34)***	***0.031***	***+3.90 (0.88, 6.93)***	***0.011***
Cerebral function-no	205 (84.36)	36.35 (35.10, 37.61) [b]		36.47 (35.21, 37.72) [b]	
Cerebral function-yes	38 (15.64)	***+5.20 (2.02, 8.39)***	***0.001***	***+4.54 (1.30, 7.78)***	***0.006***
Visual function-no	166 (70.34)	37.10 (35.68, 38.51) [b]		37.23 (35.83, 38.63) [b]	
Visual function-yes	70 (29.66)	-0.24 (-2.84, 2.37)	0.859	-0.63 (-3.22, 1.96)	0.632

Statistical significance (p<0.05) was denoted in bold and italics.
a: adjusted for sex, MS type, and study centres; b: mean ASO of the reference group

5.5.4 Smoking of tobacco and marijuana

Ever smokers had approximately 5.11-years later onset than those who never smoked ($p<0.001$) (Table 3). The magnitudes were similar between past smokers and current smokers. Cases with an earlier ASO might have had less opportunity to start smoking, so we tested this by only including cases with ASO ≥28 years (as 27 was the oldest age of taking up smoking in this cohort). While attenuating the magnitude, a history of smoking remained significantly associated with ASO (Table 3). To examine whether the results were due to an age or cohort effect, we repeated the analysis in the matched healthy controls. Each control was given the ASO of the matched cases. If the association was also observed in controls, then that is likely due to an age or cohort effect. We found no association between smoking status and ASO (never smokers vs. ever smokers: adjusted β=+0.11 years, 95% CI -1.54 - 1.77, p=0.89), suggesting the significant association in cases was not due to an age or cohort effect. Among ever smokers, the ASO was similar whether the smoking was taken up early (<16 years) or later ($p_{interaction}=0.26$), or the duration was longer or shorter ($p_{interaction}=0.94$).

Marijuana use was more common in male cases than in females (OR=0.50, p=0.020), and a history of marijuana use was positively associated with smoking (OR=8.05, $p<0.001$). A history of marijuana use was associated with a 6.03-years earlier ASO (Table 3). The magnitudes were similar between past users and current users. We next evaluated associations among cases with ASO ≥31 years (30 years was the oldest age of taking up marijuana in this cohort). Magnitudes were attenuated but still highly

significant: in this subgroup, current marijuana users had a 3.88-years earlier onset than cases without a marijuana history (Table 3).

Table 5.3Associations between smoking behaviours and age of symptom at onset

		Univariable		multivariable [a]	
	No. (%)	β (95% CI)	P	β (95% CI)	P
Smoking ever					
No	103 (37.59)	35.6 (33.79, 37.41) [b]		34.24 (32.41, 36.06) [b]	
Yes	171 (62.41)	***+2.71 (0.42, 5.00)***	***0.021***	***+5.11 (2.74, 7.48)***	***<0.001***
Smoking ever (ASO ≥ 28 years)					
No	79 (35.59)	38.71 (37.2, 40.22)		37.89 (36.45, 39.34)	
Yes	143 (64.41)	+1.79 (-0.12, 3.71)	0.073	***+3.05 (1.16, 4.95)***	***0.002***
Smoking status					
Never smoked	103 (37.59)	35.6 (33.78, 37.41) [b]		34.21 (32.38, 36.03)	
Past smokers	101 (36.86)	***+2.62 (0.04, 5.20)***	***0.047***	+4.80 (2.21, 7.38)	***<0.001***
Current smokers	70 (25.55)	+2.85 (-0.01, 5.70)	0.054	+5.66 (2.69, 8.64)	***<0.001***
Trend			***0.037***		***<0.001***
Smoking status (ASO ≥ 28years)					
Never smoked	79 (35.59)	38.71 (37.2, 40.22)		37.89 (36.44, 39.34)	
Past smokers	83 (37.39)	+2.12 (-0.05, 4.30)	0.062	***+3.05 (0.95, 5.14)***	***0.004***
Current smokers	60 (27.03)	+1.34 (-1.01, 3.68)	0.273	***+3.06 (0.65, 5.47)***	***0.013***
Trend			0.219		***0.010***
Age uptake smoking (ASO ≥ 28years)					
≥16 years	84 (58.74)	41.45 (39.85, 43.05)		41.21 (39.72, 42.7)	
<16 years	59 (41.26)	-1.77 (-4.22, 0.68)	0.162	-1.35 (-3.66, 0.97)	0.258
Duration of smoking before age 27 (ASO ≥ 28years)					
≤ 10 years	55 (40.44)	40.97 (38.99, 42.95)		40.58 (38.67, 42.5)	
>10 years	81 (59.56)	-0.51 (-3.07, 2.04)	0.693	+0.10 (-2.40, 2.60)	0.942
Marijuana ever					
No	190 (69.6)	38.58 (37.26, 39.9) [b]		39.23 (37.91, 40.55) [b]	
Yes	83 (30.4)	***-4.04 (-6.43, -1.64)***	***0.001***	-6.03 (-8.62, -3.45)	***<0.001***
Marijuana ever (ASO ≥ 31 years)					
No	151 (74.02)	41.29 (40.24, 42.34) [b]		41.5 (40.43, 42.57) [b]	
Yes	53 (25.98)	***-2.20 (-4.14, -0.27)***	***0.026***	***-2.80 (-4.89, -0.71)***	***0.009***
Marijuana status					
Never	190 (69.6)	38.58 (37.26, 39.90) [c]		38.56 (37.25, 39.87) [b]	
Past users	63 (23.08)	***-4.08 (-6.73, -1.43)***	***0.003***	***-3.98 (-6.63, -1.34)***	***0.003***
Current users	20 (7.33)	-3.92 (-8.20, 0.37)	0.070	-3.46 (-7.87, 0.94)	0.123
Trend			***0.003***		***0.004***
Marijuana status (ASO≥31 years)					
Never	151 (74.02)	41.28 (40.24, 42.33) [b]		41.25 (40.22, 42.28) [b]	
Past users	39 (19.12)	-1.50 (-3.71, 0.70)	0.175	-1.35 (-3.58, 0.88)	0.238
Current users	14 (6.86)	***-4.06 (-7.16, -0.95)***	***0.010***	***-3.88 (-7.09, -0.66)***	***0.018***
Trend			***0.008***		***0.016***

Statistical significance ($p<0.05$) was denoted in bold and italics.
a: adjusted for sex, MS type, study centresand tobacco smoking status/marijuana use status ; b: mean ASO of the reference group

5.5.5 Offspring numbers and age at menarche

Having children was strongly associated with a later ASO (Table 4). The magnitude was potentially biased since those with a later ASO may have had different opportunities to have children. We therefore chose a cut-off point of ASO that balanced sample size against the offspring number information, and by restricting to those with ASO≥31 years, we could include 204 cases and 56.9% of participant offspring. After restriction, a significant dose-dependent association remained, albeit with reduced magnitude; e.g. those with 1 child had, on average, a 1.39 year later onset, and those with ≥2 children had a 3.61-years later onset. This dose-dependent association was found in both male and female participants (test for interaction p=0.11), actually of greater magnitude in males than females. To examine whether the results were due to an age or cohort effect, we repeated the analyses in the healthy controls where the controls were assigned the same ASO as their matched cases. Having more children was also significantly associated with a later ASO in controls (0 vs. 1: β=4.55, p<0.001; 0 vs. ≥2: β=10.41, p<0.001; p for trend<0.001), and restricting to those with ASO≥31 years still showed a significant association (0 vs. 1: β=1.39, p=0.16; 0 vs. ≥2: β=3.48, p<0.001; p for trend<0.001). This suggests that the significant association of offspring number and ASO in cases was largely driven by an age or cohort effect.

The age of the participant when having their first child (≤20, 21-25, 26-30, 31-35, ≥36) was not associated with ASO; the average ASO was around 41 years for each child birth age group. There was no association between age of menarche and ASO (p=0.22), persisting after adjustment for MS onset type and study centre.

Table 5.4Associations between offspring numbers/age at menarche and age of symptom onset

		Univariable		multivariable	
	No. (%)	β (95% CI)	p	β (95% CI)	p
Offspring number [a]					
0	111 (39.78)	31.16 (29.7, 32.63) [c]		31.37 (29.9, 32.83) [c]	
1	39 (13.98)	***+5.97 (3.01, 8.92)***	***<0.001***	***+5.51 (2.57, 8.44)***	***<0.001***
2 or more	129 (46.24)	***+11.12 (9.06, 13.18)***	***<0.001***	***+10.88 (8.84, 12.93)***	***<0.001***
Trend			***<0.001***		***<0.001***

Offspring number (ASO ≥ 31 years) [b]					
0	89 (43.00)	39.25 (37.99, 40.5) [c]		39.22 (37.99, 40.45) [c]	
1	37 (17.87)	+1.67 (-0.74, 4.09)	0.182	+1.39 (-0.98, 3.77)	0.254
2 or more	81 (39.13)	***+3.42 (1.47, 5.36)***	***0.001***	***+3.61 (1.69, 5.54)***	***<0.001***
Trend			***0.001***		***<0.001***
Age of menarche [a]					
8-12 years	98 (46.23)	37.07 (35.17, 38.97) [c]		37.13 (35.24, 39.01) [c]	
13-14 years	87 (41.04)	+1.13 (-1.64, 3.9)	0.432	+0.98 (-1.8, 3.75)	0.493
15-23 years	27 (12.74)	***-4.19 (-8.24, -0.15)***	***0.042***	-3.72 (-7.76, 0.32)	0.068
Trend			0.221		0.269

Statistical significance (p<0.05) was denoted in bold and italics.
a: adjusted for sex, MS type, and study centres; b: samples limited to those with ASO ≥ age 31 years and summarizing offspring number < age 31 years, adjusted for sex, MS type, and study centres. c: mean ASO of the reference group

5.5.6 Mutually adjusted model

We next built a combined model based on the significant findings (tobacco smoking, marijuana use, MS onset type, cerebral dysfunction, and bowel & bladder dysfunction). The final model explained approximately 12% of the total variance in ASO, with marijuana use and tobacco smoking having the largest relative contributions (Table 5).

Table 5.5Mutually adjusted model including all factors associated with age of symptom at onset.

	All cases (adjusted R^2=0.12)		
	β (95% CI)	P	RC
Smoking status			5.0%
Never smoking	35.14 (33.31, 36.98)		
Past users	+3.80 (1.21, 6.40)	***0.004***	
Current users	+4.72 (1.74, 7.71)	***0.002***	
Trend		***0.001***	
Marijuana history			9.2%
Never marijuana	39.47 (38.17, 40.78)		
Past users	-5.49 (-8.29, -2.70)	***<0.001***	
Current users	-7.23 (-11.75, -2.71)	0.002	
Trend		***<0.001***	
MS onset type			0.6%
RRMS	37.58 (36.46, 38.69)		
PPMS	+2.23 (-2.05, 6.52)	0.313	
Cerebral function			5.9%
No	36.96 (35.78, 38.14)		
Yes	+4.58 (1.68, 7.48)	0.002	
Bowel & bladder function			1.2%
No	37.3 (36.09, 38.51)		
Yes	+2.13 (-0.61, 4.87)	0.130	

Statistical significance (p<0.05) was denoted in bold and italics.
RC: relative contribution
a: mean ASO of the reference group

5.5.7 Sensitivity analyses

Sensitivity analyses that restricted to cases with FDE during the study recruitment period or diagnosed MS cases did not materially alter the results (Supplementary Tables).

5.6 Discussion

In this comprehensive study, we systematically evaluated the association between ASO and established / emerging risk factors for MS onset. A model including all the significant factors explained 12% of the total variance. Of the individual risk factors, a history of tobacco smoking, progressive-onset type, and cerebral / bowel & bladder function impairment at onset were associated with a later onset, while marijuana use was associated with an earlier onset. *HLA-A2* genotype, *HLA-DR15* genotype, latitude of study site, past sun exposure, current 25(OH)D levels, history of IM, offspring number, age of menarche and sex were not associated with ASO.

A strength of our study is the detailed information on exposure variables, as well as the study neurologist interview and review of medical records for optimal assessment of the ASO. Also, our findings showed a robustness when repeating the analysis among those with diagnosed MS by 5-year review (potentially excluding some cases who may yet convert to MS after this point), as well as restricting to cases with FDE during study recruitment period (excluding some whose ASO was slightly less certain as they had had a previously unrecognised event thought to now represent CNS demyelination). A challenge was that a number of exposures were intrinsically associated with ASO (i.e. those who had a later ASO would have greater opportunity to accumulate a higher level of exposure before onset simply as a function of time). To overcome this issue, we assessed the temporal relationship between exposures and

outcome through analysing some exposure variables before a specific age and analysing the association with ASO after this point. This, however, reduced the sample size for those analyses, and also assumed that there was nothing unusual about those with a later vs earlier onset. To examine whether some observed associations were due to an age or cohort effect, we tested the existence of an effect existed in the age and sex matched controls. Based on these analyses, we believe that the between offspring and ASO was not a true association.

Another limitation was that some exposures such as IM and past exposure to sunlight happened years prior to study entry, and it was difficult for us to detect whether information about these variables was accurately recorded at baseline interview. IM was decoded into two ways (those who did not know whether they had an IM before was decoded as no history of IM and missing) to decrease the influence of the potential error of recall. We used lifetime calendar to measure the past exposure to sunlight, which has been suggested to be valid by some research with the polysulphone badge readings as the comparison method[41]. Influence of potential recall error was extremely difficult of be controlled for fully, however, there was no evidence that the potential recall error of these variables was systematically differential by age at onset, and the significant association between these exposures and MS onset/relapses suggested that the influence of the potential error of recall was minor on the results.

Age at onset in Ausimmune study was measured and reviewed by experienced neurologists. However, we still could not determine that no measurement error existed in the study especially in the progressive-onset cases. We did some sensitivity

analysis in the relapsing-onset cases with date of FDE during recruitment period, which showed a similar association with our primary analysis. Moreover, there was no evidence that the potential measurement error of ASO was differential with the exposures of interest and a biased ASO would have reduced the association towards null. We therefore believed that the potential measurement error of ASO would not influence the results martially.

In agreement with previous studies[17 20 21], progressive-onset patients showed a later ASO than relapsing-onset patients. In this study, patients with initial presentation of cerebral or bowel & bladder function impairment showed a 4.4 and 3.5 years later ASO, respectively. This is partly in contrast to a previous study[22], which found no association between psychiatric (p=0.31) or cognitive (p=0.93) symptoms and ASO, while a positive relationship between sphincteric symptoms and ASO was shown (p=0.012). Notably there were marked differences in the ASO between these studies – roughly 37 years in the current study compared to approximately 30 years – and this may account for the divergent findings.

Despite tobacco smoking being typically shown to be a risk factor for FCD, including in the Ausimmune Study[5], in this study tobacco smoking delayed the average ASO by 4 years in our multivariable model. Two other studies have also shown a similar direction of the association with our study, magnitude 2.60 years (n=7,883)[23] and 0.82 years (n=540)[11], respectively. A null association between tobacco smoking and ASO in controls supported that this association was not due to an age or cohort effect. A good explanation for the opposite relationship for tobacco use in MS onset [24] versus ASO[11 23] is challenging. It could relate to the anti-inflammatory effects of nicotine

upon T-cells, B-cells and even dendritic cells[25]. It is possible that tobacco smoking more impact more on neurodegeneration rather than immune dysfunction[26] and older onset cases may have a greater neurodegenerative component with a faster progression[27].

Marijuana use was associated with a 6.0-years earlier ASO, despite our previous findings from the Ausimmune Study[5] showing no association between marijuana use and MS risk (never vs. ever, adjusted OR 0.91 (0.84-1.54)). Other research has also demonstrated a detrimental effect of marijuana on the CNS. Two studies[28 29] have showed that marijuana use was associated with poorer cognitive performance in MS patients. Thus there is some epidemiological evidence in support of potential deleterious impacts of marijuana use in MS, though the means by which this occurs and why its impact is deleterious but that of tobacco is not, requires further study.

Consistent with previous research[10 11 15 30], we found no association between sex, history of infectious mononucleosis, *HLA-A2* or *HLA-DR15* genotype and ASO. Latitude of study site, deseasonalised 25(OH)D levels and UVR exposures were not associated with ASO in the present study. Using the large MSBase global dataset (n=22,162), we had previously found an inverse association between latitude and ASO (every 10 ºncrease in latitude was associated with a 0.82-year earlier onset, $p=2.20x10^{-13}$)[15]. However, in line with the present study, subgroup analysis restricting to Australian participants showed a non-significant association. We were unclear why this latitudinal gradient was not found in Australia, and we assumed that some difference between Europe and Australia in temperature, culture/lifestyle, genetics[16] may underlie the disparate findings. Our previous study also found that increasing

UVR exposure from age 6-15 years or lifetime actinic damage was not associated with ASO[31]. Another cross-sectional study[10] of 1,161 MS patients found that vitamin D-associated SNPs and vitamin D supplementation (multivitamin/vitamin D or fatty fish) were not associated with ASO.

The disparate findings in different outcomes (ASO, risk of MS, and progression measures such as relapses) were not unexpected. While there might be an increased likelihood for a factor to be related with multiple outcomes, this should not be seen to be "in contrast" with the literature, and therefore does not invalidate our findings. For example, EBV has been significantly associated with MS onset but no determined effect has been made with MS progression. We therefore prefer limit to the literature to factors associated with ASO.

For a study with participants enrolled from different centres, the influence of spectrum effect was considered in the analysis. In Ausimmune study, all cases were diagnosed with a standardised protocol by experienced neurologists, and adjustment for study centre was performed in every multivariable model, so we believed that spectrum bias would not influence the results materially.

While we identified some factors that were associated with ASO, we only explained 12% of the total variance and thus leaving the majority unexplained. We demonstrated that marijuana use was associated with an earlier MS onset, in line with evidence indicating its deleterious impacts on the CNS, and that a history of tobacco smoking was associated with a later ASO. Further research is required to better understand these opposite effects and underlying pathways. These results, if

corroborated and supported in other studies, may aid in the understanding of MS and potentially contribute to delaying MS onset.

5.7 Postscript

Chapter 4 and 5 discussed association between environmental/behavioural factors and age of symptom onset in MS patients. The next chapter will investigate association between viral infections and MS clinical course.

5.8 Reference

1. Mechelli R, Annibali V, Ristori G, et al. Multiple sclerosis etiology: beyond genes and environment. *Expert review of clinical immunology* 2010;6(3):481-90. doi: 10.1586/eci.10.11
2. Haines JL, Terwedow HA, Burgess K, et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group.*Human molecular genetics* 1998;7(8):1229-34.
3. Munger KL, Levin LI, Hollis BW, et al. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA : the journal of the American Medical Association* 2006;296(23):2832-8. doi: 10.1001/jama.296.23.2832
4. Lucas RM, Ponsonby AL, Dear K, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011;76(6):540-8. doi: 10.1212/WNL.0b013e31820af93d
5. Ponsonby AL, Lucas RM, Dear K, et al. The physical anthropometry, lifestyle habits and blood pressure of people presenting with a first clinical demyelinating event compared to controls: the Ausimmune study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(13):1717-25. doi: 10.1177/1352458513483887
6. Tao C, Simpson Jr S, Taylor BV, et al. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. *Journal of the neurological sciences* 2017;372:239-49. doi: http://dx.doi.org/10.1016/j.jns.2016.11.060
7. van der Mei I, Lucas RM, Taylor BV, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(4):461-9. doi: 10.1177/1352458515594040 [published Online First: 2015/07/23]
8. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011;476(7359):214-9. doi: 10.1038/nature10251
9. Ponsonby AL, Lucas RM, van der Mei IA, et al. Offspring number, pregnancy, and risk of a first clinical demyelinating event: the AusImmune Study. *Neurology*

2012;78(12):867-74. doi: 10.1212/WNL.0b013e31824c4648
10. Laursen JH, Sondergaard HB, Sorensen PS, et al. Association between age at onset of multiple sclerosis and vitamin D level-related factors. *Neurology* 2016;86(1):88-93. doi: 10.1212/wnl.0000000000002075 [published Online First: 2015/10/09]
11. McDowell TY, Amr S, Culpepper WJ, et al. Sun exposure, vitamin D and age at disease onset in relapsing multiple sclerosis. *Neuroepidemiology* 2011;36(1):39-45. doi: 10.1159/000322512
12. Manouchehrinia A, Tench CR, Maxted J, et al. Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain : a journal of neurology* 2013;136(Pt 7):2298-304. doi: 10.1093/brain/awt139 [published Online First: 2013/06/13]
13. Masterman T, Ligers A, Olsson T, et al. HLA-DR15 is associated with lower age at onset in multiple sclerosis. *Annals of neurology* 2000;48(2):211-9.
14. Barcellos LF, Sawcer S, Ramsay PP, et al. Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. *Human molecular genetics* 2006;15(18):2813-24. doi: 10.1093/hmg/ddl223 [published Online First: 2006/08/15]
15. Smestad C, Brynedal B, Jonasdottir G, et al. The impact of HLA-A and -DRB1 on age at onset, disease course and severity in Scandinavian multiple sclerosis patients. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2007;14(8):835-40. doi: 10.1111/j.1468-1331.2007.01825.x
16. Moutsianas L, Jostins L, Beecham AH, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nature genetics* 2015;47(10):1107-13. doi: 10.1038/ng.3395 [published Online First: 2015/09/08]
17. Tao C, Simpson S, Jr., van der Mei I, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2016 doi: 10.1136/jnnp-2016-314013
18. Lucas R, Ponsonby AL, McMichael A, et al. Observational analytic studies in multiple sclerosis: controlling bias through study design and conduct. The Australian Multicentre Study of Environment and Immune Function.*Multiple sclerosis (Houndmills, Basingstoke, England)* 2007;13(7):827-39. doi: 10.1177/1352458507077174 [published Online First: 2007/09/21]
19. Pan G, Simpson S, Jr., van der Mei I, et al. Role of genetic susceptibility variants in predicting clinical course in multiple sclerosis: a cohort study. *Journal of neurology, neurosurgery, and psychiatry* 2016;87(11):1204-11. doi: 10.1136/jnnp-2016-313722 [published Online First: 2016/08/26]
20. Stankoff B, Mrejen S, Tourbah A, et al. Age at onset determines the occurrence of the progressive phase of multiple sclerosis. *Neurology* 2007;68(10):779-81. doi: 10.1212/01.wnl.0000256732.36565.4a
21. Confavreux C, Vukusic S. Natural history of multiple sclerosis: a unifying concept. *Brain : a journal of neurology* 2006;129(Pt 3):606-16. doi: 10.1093/brain/awl007
22. Cossburn M, Ingram G, Hirst C, et al. Age at onset as a determinant of presenting phenotype and initial relapse recovery in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(1):45-54. doi:

10.1177/1352458511417479
23. Hedstrom AK, Hillert J, Olsson T, et al. Smoking and multiple sclerosis susceptibility. *European journal of epidemiology* 2013;28(11):867-74. doi: 10.1007/s10654-013-9853-4 [published Online First: 2013/10/23]
24. Hedstrom AK, Hillert J, Olsson T, et al. Nicotine might have a protective effect in the etiology of multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(8):1009-13. doi: 10.1177/1352458512471879 [published Online First: 2013/01/16]
25. Filippini P, Cesario A, Fini M, et al. The Yin and Yang of non-neuronal alpha7-nicotinic receptors in inflammation and autoimmunity.*Current drug targets* 2012;13(5):644-55. [published Online First: 2012/02/04]
26. Pittas F, Ponsonby AL, van der Mei IA, et al. Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis. *Journal of neurology* 2009;256(4):577-85. doi: 10.1007/s00415-009-0120-2
27. Leray E, Yaouanq J, Le Page E, et al. Evidence for a two-stage disability progression in multiple sclerosis. *Brain : a journal of neurology* 2010;133(Pt 7):1900-13. doi: 10.1093/brain/awq076 [published Online First: 2010/04/29]
28. Honarmand K, Tierney MC, O'Connor P, et al. Effects of cannabis on cognitive function in patients with multiple sclerosis. *Neurology* 2011;76(13):1153-60. doi: 10.1212/WNL.0b013e318212ab0c
29. Pavisian B, MacIntosh BJ, Szilagyi G, et al. Effects of cannabis on cognition in patients with MS: a psychometric and MRI study. *Neurology* 2014;82(21):1879-87. doi: 10.1212/WNL.0000000000000446
30. Wu JS, Qiu W, Castley A, et al. Modifying effects of HLA-DRB1 allele interactions on age at onset of multiple sclerosis in Western Australia. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(1):15-20. doi: 10.1177/1352458509350312
31. van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *Bmj* 2003;327(7410):316. doi: 10.1136/bmj.327.7410.316

5.9 Supplementary Tables

Table 5.6 Sensitivity analysis of sex, IM history, *HLA-DR15* genotype, *HLA-A2* genotype of the total cohort in the AusImmune Study by restricting to cases with FDE during recruitment period and those with diagnosed MS before 5-year review

	Primary analysis		Restricted to cases with FDE during recruitment period		Restricted to MS	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
Sex						
Male	37.21 (34.90, 39.52) [a]		37.16 (34.58, 39.74) [a]		37.00 (34.08, 39.93) [a]	
Female	+0.15 (-2.49, 2.80)	0.913	-0.52 (-3.48, 2.43)	0.725	-0.03 (-3.29, 3.24)	0.992
Past history of IM						
No	37.7 (36.41, 39.00) [a]		37.03 (35.60, 38.46) [a]		37.59 (36.09, 39.08) [a]	
Yes	-1.33 (-3.83, 1.18)	0.302	-1.07 (-3.85, 1.71)	0.449	-1.91 (-4.80, 0.98)	0.193
HLA-DR15 (rs9271366)						
AA	36.89 (34.91, 38.86) [a]		36.06 (33.93, 38.20) [a]		36.99 (34.60, 39.38) [a]	
AG	+1.42 (-1.26, 4.0 9)	0.303	+2.11 (-0.82, 5.03)	0.163	+0.53 (-2.65, 3.72)	0.739
GG	+4.74 (-2.43, 11.91)	0.202	+3.14 (-4.59, 10.87)	0.432	+4.55 (-2.94, 12.05)	0.225
Trend		0.151		0.144		0.376
HLA-A2 (rs2844821)						
AA	37.17 (35.43, 38.92) [a]		36.69 (34.78, 38.59) [a]		36.84 (34.85, 38.83) [a]	
AG	+1.38 (-1.24, 4.01)	0.298	+1.32 (-1.57, 4.20)	0.365	+1.52 (-1.55, 4.59)	0.332

Statistical significance ($p<0.05$) was denoted in bold and italics.
adjusted for sex, MS type, and study centresa: mean ASO of the reference group

Table 5.7 Sensitivity analysis of UV exposure of the total cohort in the AusImmune Study by restricting to cases with FDE during recruitment period and those with diagnosed MS before 5-year review

	Primary analysis	Restricted to cases with FDE during recruitment period	Restricted to MS

	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
Winter UV dose within 5 years before onset [b]						
0-0.27 x10^2 kJ/m^2	38.06 (35.73, 40.40) [a]		38.07 (35.5, 40.64) [a]		37.92 (35.44, 40.41) [a]	
>0.27-0.45 x10^2 kJ/m^2	-1.33 (-4.54, 1.88)	0.423	-2.00 (-5.54, 1.53)	0.273	-2.89 (-6.49, 0.72)	0.124
>0.45-0.75 x10^2 kJ/m^2	-2.16 (-5.44, 1.13)	0.202	-1.68 (-5.30, 1.94)	0.363	-2.62 (-6.26, 1.03)	0.163
>0.75-3.16 x10^2 kJ/m^2	+0.17 (-3.13, 3.46)	0.923	-1.51 (-5.20, 2.18)	0.424	+0.86 (-2.80, 4.52)	0.649
Trend		0.974		0.478		0.718
Winter UV dose within 10 years before onset [b]						
0-0.58 x10^2 kJ/m^2	38.36 (36.08, 40.64) [a]		38.76 (36.2, 41.31) [a]		38.21 (35.76, 40.65) [a]	
>0.58 - 0.95x10^2 kJ/m^2	-2.07 (-5.29, 1.14)	0.205	-3.16 (-6.7, 0.38)	0.082	-3.42 (-7.05, 0.21)	0.073
>0.95 - 1.59x10^2 kJ/m^2	-2.21 (-5.45, 1.03)	0.183	-2.62 (-6.24, 0.99)	0.155	-2.84 (-6.40, 0.72)	0.124
>1.59 - 5.26 x10^2 kJ/m^2	-0.28 (-3.51, 2.96)	0.868	-2.18 (-5.82, 1.46)	0.239	+0.38 (-3.25, 4.01)	0.843
Trend		0.848		0.305		0.919
Winter UV dose within 15 years before onset [b]						
0-0.93 x10^2 kJ/m^2	39.68 (37.47, 41.9) [c]		39.07 (36.6, 41.53) [a]		38.88 (36.48, 41.27) [a]	
>0.93 - 1.59x10^2 kJ/m^2	***-3.37 (-6.46, -0.28)***	***0.032***	-1.86 (-5.31, 1.58)	0.286	-3.79 (-7.21, -0.36)	***0.033***
>1.59 - 2.51x10^2 kJ/m^2	-2.06 (-5.22, 1.09)	0.203	-2.95 (-6.39, 0.48)	0.092	-2.66 (-6.27, 0.94)	0.154
>2.51 - 8.47x10^2 kJ/m^2	-1.37 (-4.50, 1.76)	0.389	-2.29 (-5.78, 1.2)	0.201	+0.14 (-3.35, 3.64)	0.939
Trend		0.568		0.153		0.847
Summer UV dose within 5 years before onset [b]						
0-0.45 x10^2 kJ/m^2	37.08 (34.79, 39.38) [a]		36.54 (33.85, 39.22) [a]		36.93 (34.49, 39.37) [a]	
>0.45 - 0.62x10^2 kJ/m^2	-0.69 (-3.90, 2.52)	0.681	-0.34 (-4.01, 3.33)	0.861	-0.66 (-4.18, 2.86)	0.713
>0.62 - 0.72x10^2 kJ/m^2	-1.13 (-4.36, 2.09)	0.492	-1.30 (-4.90, 2.30)	0.476	-2.13 (-5.7, 1.45)	0.239
>0.72 - 0.95x10^2 kJ/m^2	2.48 (-0.86, 5.83)	0.153	+2.49 (-1.32, 6.30)	0.202	2.83 (-0.98, 6.63)	0.148
Trend		0.208		0.303		0.365

Summer UV dose within 10 years before onset [b]						
0-3.28 x10^2 kJ/m^2	38.40 (36.13, 40.67) [a]		37.82 (35.18, 40.46) [a]		38.60 (36.17, 41.03) [a]	
>3.28 - 4.92x10^2 kJ/m^2	-3.27 (-6.44, -0.11)	0.043	-3.17 (-6.74, 0.39)	0.078	***-3.88 (-7.35, -0.41)***	***0.028***
>4.92 - 6.80x10^2 kJ/m^2	-2.66 (-5.84, 0.52)	0.095	-2.72 (-6.34, 0.89)	0.142	***-4.13 (-7.57, -0.68)***	***0.019***
>6.80 - 9.53x10^2 kJ/m^2	+1.36 (-1.97, 4.69)	0.423	+1.59 (-2.17, 5.35)	0.413	+1.80 (-2.04, 5.64)	0.363
Trend		0.454		0.382		0.762
Summer UV dose within 15 years before onset [b]						
0-5.53 x10^2 kJ/m^2	39.82 (37.66, 41.97) [c]		38.49 (35.91, 41.07) [a]		39.49 (37.16, 41.82) [a]	
>5.53 - 7.37x10^2 kJ/m^2	***-3.91 (-6.91, -0.91)***	***0.011***	***-3.53 (-6.91, -0.16)***	***0.040***	***-4.40 (-7.72, -1.08)***	***0.009***
>7.37 - 9.99x10^2 kJ/m^2	***-3.95 (-6.95, -0.95)***	***0.010***	***-3.43 (-6.86, 0.01)***	***0.050***	***-5.35 (-8.62, -2.09)***	***0.001***
>9.99 - 18.99x10^2 kJ/m^2	+0.38 (-2.66, 3.42)	0.810	+2.12 (-1.57, 5.80)	0.263	+2.45 (-1.25, 6.16)	0.187
Trend		0.812		0.262		0.729
UV dose within 5 years before onset [b]						
0-2.02 x10^2 kJ/m^2	37.07 (34.77, 39.37) [a]		36.36 (33.70, 39.01) [a]		36.89 (34.43, 39.34) [a]	
>2.02 - 3.07x10^2 kJ/m^2	-0.95 (-4.17, 2.27)	0.562	-0.33 (-3.92, 3.27)	0.858	-1.25 (-4.83, 2.33)	0.503
>3.07 - 4.03x10^2 kJ/m^2	-0.20 (-3.44, 3.04)	0.903	+0.18 (-3.50, 3.85)	0.929	-1.15 (-4.76, 2.45)	0.529
>4.03 - 7.82x10^2 kJ/m^2	+1.82 (-1.54, 5.18)	0.286	+1.73 (-2.11, 5.56)	0.381	+2.14 (-1.63, 5.92)	0.268
Trend		0.265		0.369		0.359
UV dose within 10 years before onset [b]						
0-4.38 x10^2 kJ/m^2	37.74 (35.41, 40.08) [a]		37.04 (34.30, 39.78) [a]		37.61 (35.05, 40.16) [a]	
>4.38 - 6.07x10^2 kJ/m^2	-2.49 (-5.68, 0.71)	0.132	-1.96 (-5.55, 1.63)	0.278	-2.56 (-6.10, 0.97)	0.158
>6.07 - 8.02x10^2 kJ/m^2	-0.92 (-4.19, 2.34)	0.581	-0.38 (-4.11, 3.35)	0.839	-1.95 (-5.59, 1.70)	0.303
>8.02 - 14.50x10^2 kJ/m^2	+1.49 (-1.87, 4.85)	0.387	+1.39 (-2.48, 5.27)	0.475	+1.76 (-2.09, 5.62)	0.372
Trend		0.265		0.318		0.429
UV dose within 15 years						

before onset [b]						
0-6.87 x10^2 kJ/m^2	39.35 (37.18, 41.52) [c]		38.70 (36.19, 41.20) [a]		38.94 (36.54, 41.34) [a]	
>6.87 - 9.30x10^2 kJ/m^2	***-3.13 (-6.15, -0.10)***	***0.043***	***-3.99 (-7.32, -0.66)***	***0.019***	***-3.51 (-6.90, -0.13)***	***0.042***
>9.30 - 12.00x10^2 kJ/m^2	***-3.42 (-6.45, -0.38)***	***0.027***	***-3.41 (-6.80, -0.02)***	***0.049***	***-4.14 (-7.67, -0.61)***	***0.021***
>12.00 - 22.46x10^2 kJ/m^2	+0.85 (-2.21, 3.91)	0.587	+1.80 (-1.84, 5.44)	0.333	+1.26 (-2.37, 4.90)	0.503
Trend		0.643		0.382		0.738
Winter UV dose between 6-15 [b]						
0-0.71x10^2 kJ/m^2	37.83 (35.53, 40.13) [a]		37.53 (34.94, 40.12) [a]		36.91 (34.33, 39.49) [a]	
>0.71-1.12 x10^2 kJ/m^2	+0.06 (-3.19, 3.30)	0.965	+0.20 (-3.43, 3.82)	0.924	+1.20 (-2.50, 4.91)	0.521
>1.12-2.26 x10^2 kJ/m^2	-1.13 (-4.36, 2.11)	0.487	-1.19 (-4.79, 2.40)	0.523	-1.05 (-4.79, 2.69)	0.583
>2.26-7.68 x10^2 kJ/m^2	-1.21 (-4.48, 2.05)	0.468	-1.92 (-5.54, 1.70)	0.296	-0.57 (-4.22, 3.09)	0.758
Trend		0.362		0.223		0.518
Summer UV dose between 6-15 [b]						
0-4.53 x10^2 kJ/m^2	37.19 (34.9, 39.48) [a]		35.81 (33.27, 38.36) [a]		37.44 (34.97, 39.91) [a]	
>4.53-6.27 x10^2 kJ/m^2	-0.42 (-3.64, 2.80)	0.802	+0.83 (-2.77, 4.44)	0.648	-2.18 (-5.79, 1.44)	0.243
>6.27-7.23 x10^2 kJ/m^2	+1.49 (-1.73, 4.70)	0.363	+2.79 (-0.72, 6.29)	0.123	+0.49 (-3.12, 4.10)	0.793
>7.23-7.99 x10^2 kJ/m^2	-0.83 (-4.13, 2.47)	0.624	+0.06 (-3.63, 3.76)	0.968	-0.98 (-4.75, 2.79)	0.612
Trend		0.959		0.628		0.957
Annual UV dose between 6-15 [b]						
0-5.64x10^2 kJ/m^2	37.18 (34.88, 39.47) [a]		36.51 (33.92, 39.10) [a]		37.16 (34.64, 39.68) [a]	
>5.64-7.39 x10^2 kJ/m^2	+0.40 (-2.83, 3.63)	0.812	+0.50 (-3.10, 4.10)	0.787	-0.58 (-4.18, 3.03)	0.748
>7.39-9.19 x10^2 kJ/m^2	-0.39 (-3.63, 2.85)	0.813	+0.14 (-3.48, 3.77)	0.939	-0.94 (-4.68, 2.81)	0.619
>9.19-10.87 x10^2 kJ/m^2	+0.27 (-3.03, 3.56)	0.868	+0.33 (-3.34, 4.00)	0.864	+0.05 (-3.68, 3.78)	0.979
Trend		1.000		0.913		0.953

Statistical significance ($p<0.05$) was denoted in bold and italics.

adjusted for sex, MS type, and buttock melanin density a: mean ASO of the reference group

Table 5.8 Sensitivity analysis of MS type/initial clinical symptoms of the total cohort in the AusImmune Study by restricting to cases with FDE during recruitment period and those with diagnosed MS before 5-year review

	Multivariable analysis		**Restricted to cases with FDE during recruitment period**		**Restricted to MS**	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
MS onset type						
Relapsing-onset	36.95 (35.8, 38.09) [b]				36.52 (35.18, 37.86) [b]	
Progressive-onset	***+5.61 (1.17, 10.05)***	***0.013***			***+5.91 (1.34, 10.47)***	***0.011***
Onset symptoms						
Pyramid function-no	37.86 (36.33, 39.38) [b]		37.15 (35.53, 38.77) [b]		37.82 (35.98, 39.67) [b]	
Pyramid function-yes	-0.52 (-2.94, 1.90)	0.672	-0.15 (-2.83, 2.52)	0.91	-0.89 (-3.66, 1.88)	0.534
Cerebellar function-no	37.01 (35.66, 38.37) [b]		36.38 (34.93, 37.83) [b]		36.86 (35.23, 38.48) [b]	
Cerebellar function-yes	+2.52 (-0.08, 5.12)	0.057	***+3.45 (0.44, 6.45)***	***0.025***	+1.99 (-0.91, 4.88)	0.178
Brainstem function-no	37.4 (36.06, 38.75) [b]		36.88 (35.38, 38.37) [b]		37.28 (35.69, 38.87) [b]	
Brainstem function-yes	0.15 (-2.59, 2.89)	0.920	+0.16 (-2.86, 3.19)	0.92	-0.57 (-3.73, 2.60)	0.729
Sensory function-no	37.56 (35.81, 39.3) [b]		36.78 (34.89, 38.68) [b]		37.91 (35.85, 39.96) [b]	
Sensory function-yes	-0.33 (-2.70, 2.05)	0.791	+0.12 (-2.52, 2.75)	0.93	-1.35 (-4.12, 1.43)	0.337
Bowel & Bladder function-no	36.85 (35.58, 38.12) [b]		36.36 (34.98, 37.75) [b]		36.52 (35.02, 38.02) [b]	
Bowel & Bladder function-yes	***+3.49 (0.63, 6.34)***	***0.017***	***+3.62 (0.33, 6.91)***	***0.031***	***+3.59 (0.27, 6.91)***	***0.034***
Cerebral function-no	36.87 (35.64, 38.1) [b]		36.39 (35.03, 37.76) [b]		36.6 (35.16, 38.04) [b]	
Cerebral function-yes	***+4.37 (1.28, 7.46)***	***0.006***	***+4.19 (0.69, 7.69)***	***0.019***	***+4.58 (1.10, 8.06)***	***0.010***
Visual function-no	37.74 (36.38, 39.10) [b]		37.16 (35.62, 38.69) [b]		37.41 (35.85, 38.98) [b]	
Visual function-yes	-1.00 (-3.54, 1.54)	0.439	-0.61 (-3.37, 2.14)	0.66	-1.11 (-4.22, 2.00)	0.487
Onset symptoms in relapsing-onset cases						
Pyramid function-no	37.35 (35.84, 38.86) [b]				37.11 (35.27, 38.95) [b]	
Pyramid function-yes	-0.36 (-2.80, 2.09)	0.773			-0.65 (-3.47, 2.17)	0.652
Cerebellar function-yes	36.55 (35.19, 37.91) [b]				36.21 (34.56, 37.85) [b]	
Cerebellar function-no	***+2.91 (0.21, 5.60)***	***0.034***			+2.42 (-0.61, 5.45)	0.123
Brainstem function-no	36.78 (35.41, 38.16) [b]				36.47 (34.82, 38.11) [b]	

Brainstem function-yes	+1.14 (-1.61, 3.90)	0.418	+0.62 (-2.58, 3.81)	0.714
Sensory function-no	37.12 (35.33, 38.91) [b]		37.37 (35.23, 39.5) [b]	
Sensory function-yes	-0.21 (-2.63, 2.21)	0.866	-1.27 (-4.14, 1.59)	0.378
Bowel & Bladder function-no	36.43 (35.16, 37.71) [b]		35.93 (34.41, 37.45) [b]	
Bowel & Bladder function-yes	***+3.90 (0.88, 6.93)***	***0.011***	***+4.24 (0.62, 7.86)***	***0.022***
Cerebral function-no	36.47 (35.21, 37.72) [b]		36.05 (34.56, 37.53) [b]	
Cerebral function-yes	***+4.54 (1.30, 7.78)***	***0.006***	***+4.89 (1.20, 8.59)***	***0.009***
Visual function-no	37.23 (35.83, 38.63) [b]		36.73 (35.1, 38.37) [b]	
Visual function-yes	-0.63 (-3.22, 1.96)	0.632	-0.65 (-3.86, 2.56)	0.686

Statistical significance (p<0.05) was denoted in bold and italics.
a: adjusted for sex, MS type, and study centres; b: mean ASO of the reference group

Table 5.9 Sensitivity analysis of smoking behaviours of the total cohort in the AusImmune Study by restricting to cases with FDE during recruitment period and those with diagnosed MS before 5-year review

	Primary analysis		Restricted to cases with FDE during recruitment period		Restricted to MS	
	β (95% CI)	P			β (95% CI)	P
Smoking ever [a]						
No	35.6 (33.79, 37.41) [b]		33.48 (31.48, 35.48) [c]		34.62 (32.46, 36.78) [c]	
Yes	***+2.71 (0.42, 5.00)***	***0.021***	***+5.42 (2.80, 8.04)***	***<0.001***	***+3.99 (1.17, 6.82)***	***0.005***
Smoking ever [b]						
No	38.71 (37.2, 40.22)		37.1 (35.54, 38.66) [c]		39.1 (37.34, 40.86) [c]	
Yes	+1.79 (-0.12, 3.71)	0.073	***+3.21 (1.14, 5.28)***	***0.002***	+1.63 (-0.63, 3.90)	0.158
Smoking status [a]						
Never smoked	35.6 (33.78, 37.41) [b]		33.44 (31.44, 35.45) [c]		34.61 (32.44, 36.77) [c]	
Past smokers	***+2.62 (0.04, 5.20)***	***0.047***	***+4.80 (1.91, 7.69)***	***0.001***	***+3.84 (0.77, 6.90)***	***0.014***
Current smokers	+2.85 (-0.01, 5.70)	0.054	***+6.36 (3.14, 9.58)***	***<0.001***	***+4.28 (0.73, 7.84)***	***0.018***
Trend		***0.037***		***<0.001***		***0.013***
Smoking status [b]						

Never smoked		38.71 (37.2, 40.22)		37.09 (35.53, 38.64) [c]		39.13 (37.37, 40.89) [c]	
Past smokers		+2.12 (-0.05, 4.30)	0.062	***+2.85 (0.52, 5.17)***	***0.016***	+1.94 (-0.54, 4.42)	0.133
Current smokers		+1.34 (-1.01, 3.68)	0.273	***+3.73 (1.14, 6.31)***	***0.005***	+1.09 (-1.75, 3.93)	0.451
	Trend		0.219		***0.005***		***0.392***
Age of smoking begin [b]							
≥16		41.45 (39.85, 43.05)		39.82 (38.18, 41.46) [c]		40.92 (39.24, 42.59) [c]	
<16		-1.77 (-4.22, 0.68)	0.162	+0.22 (-2.36, 2.81)	0.873	-1.17 (-3.81, 1.48)	0.392
Duration of smoking before 28 [b]							
≤ 10 years		40.97 (38.99, 42.95)		39.64 (37.47, 41.82) [c]		40.12 (37.96, 42.29) [c]	
>10		-0.51 (-3.07, 2.04)	0.693	+0.37 (-2.42, 3.16)	0.803	+0.73 (-2.09, 3.55)	0.614
Marijuana ever [a]							
No		38.58 (37.26, 39.9) [b]		38.72 (37.27, 40.17) [c]		38.63 (37.11, 40.15) [c]	
Yes		***-4.04 (-6.43, -1.64)***	***0.001***	***-5.79 (-8.52, -3.07)***	***<0.001***	***-5.25 (-8.33, -2.18)***	***0.001***
Marijuana status [a]							
Never		38.58 (37.26, 39.90) [c]		38.74 (37.28, 40.19) [c]		38.62 (37.09, 40.14) [c]	
Past users		***-4.08 (-6.73, -1.43)***	***0.003***	***-5.58 (-8.51, -2.65)***	***<0.001***	***-5.43 (-8.74, -2.12)***	***0.001***
Current users		-3.92 (-8.20, 0.37)	0.070	***-6.55 (-11.18, -1.92)***	***0.006***	-4.58 (-10.09, 0.93)	0.102
	Trend		***0.003***		***<0.001***		***0.003***
Marijuana ever after 31							
No		41.29 (40.24, 42.34) [b]		41.07 (39.9, 42.24) [c]		41.51 (40.32, 42.70) [c]	
Yes		***-2.20 (-4.14, -0.27)***	***0.026***	***-3.33 (-5.46, -1.21)***	***0.002***	-1.95 (-4.44, 0.53)	0.118
Marijuana status after 31							
Never		41.28 (40.24, 42.33) [b]		41.08 (39.91, 42.25) [c]		41.49 (40.31, 42.68) [c]	
Past users		-1.50 (-3.71, 0.70)	0.175	***-2.89 (-5.24, -0.54)***	***0.016***	-1.09 (-3.87, 1.68)	0.439
Current users		***-4.06 (-7.16, -0.95)***	***0.010***	***-4.57 (-7.80, -1.34)***	***0.006***	***-4.30 (-8.14, -0.47)***	***0.028***
	Trend		***0.008***		***0.001***		***0.041***

Statistical significance ($p<0.05$) was denoted in bold and italics.

a:adjusted for sex, MS type, study centres, and tobacco smoking status/marijuana use status; b:exclude those with ASO<28 years old, adjust for sex, MS type, study centres, and tobacco smoking status/marijuana use status. c: mean ASO of the reference group

Table 5.10 Sensitivity analysis of offspring number/age at menarche of the total cohort in the AusImmune Study by restricting to participants with FDE during recruitment period and those with diagnosed MS before 5-year review

	Primary analysis		**Restricted to cases with FDE during recruitment period**		**Restricted to MS**	
Offspring number [a]	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
0	31.37 (29.9, 32.83) [d]		31.57 (29.95, 33.19) [d]		31.07 (29.37, 32.77) [d]	
1	***+5.51 (2.57, 8.44)***	***<0.001***	***+4.29 (1.01, 7.56)***	***0.010***	+6.39 (3.06, 9.71)	***<0.001***
2 or more	***+10.88 (8.84, 12.93)***	***<0.001***	+10.06 (7.74, 12.38)	***<0.001***	+10.99 (8.58, 13.39)	***<0.001***
Trend		***<0.001***		***<0.001***		***<0.001***
Offspring number [b]						
0	39.22 (37.99, 40.45) [d]		38.85 (37.54, 40.15) [d]		40.05 (38.59, 41.51) [d]	
1	+1.39 (-0.98, 3.77)	0.254	+0.66 (-1.91, 3.22)	0.623	+1.51 (-1.36, 4.38)	0.298
2 or more	***+3.61 (1.69, 5.54)***	***<0.001***	***+3.52 (1.37, 5.67)***	***0.001***	+2.17 (-0.05, 4.39)	0.062
Trend		***<0.001***		***0.002***		0.062
Age of menarche [c]						
8-14	37.60 (36.24, 38.96) [d]		37.14 (35.60, 38.68) [d]		37.44 (35.92, 38.97) [d]	
15-23	***-4.16 (-8.00, -0.33)***	***0.033***	***-5.95 (-10.09, -1.81)***	***0.005***	***-5.03 (-9.46, -0.60)***	***0.026***

Statistical significance ($p<0.05$) was denoted in bold and italics.
a: adjusted for sex, MS type, study centres; b: restricted to those with ASO greater than 31 and summarizing offspring number before age 31, adjust for sex, MS type, study centres; c: adjust for MS type, study centres
d: mean ASO of the reference group

5.10 Publication in chapter 5

frontiers in Neurology

ORIGINAL RESEARCH
published: 08 June 2018
doi: 10.3389/fneur.2018.00418

Onset Symptoms, Tobacco Smoking, and Progressive-Onset Phenotype Are Associated With a Delayed Onset of Multiple Sclerosis, and Marijuana Use With an Earlier Onset

*Chunrong Tao[1], Steve Simpson Jr.[1,2], Bruce V. Taylor[1], Leigh Blizzard[1], Robyn M. Lucas[3], Anne-Louise Ponsonby[4], Simon Broadley[5], AusLong/Ausimmune Investigators Group and Ingrid van der Mei[1]**

[1] Menzies Institute for Medical Research, University of Tasmania, Hobart, TAS, Australia, [2] Institute for Health & Ageing, Australian Catholic University, Melbourne, VIC, Australia, [3] National Centre for Epidemiology and Population Health, Canberra, ACT, Australia, [4] Murdoch Children's Research Institute, University of Melbourne, Melbourne, VIC, Australia, [5] School of Medicine, Griffith University, Gold Coast, QLD, Australia

OPEN ACCESS

Edited by:
Robert Weissert,
University of Regensburg, Germany

Reviewed by:
Maria José Sá,
Centro Hospitalar São João, Portugal
Moussa Antoine Chalah,
Hôpitaux Universitaires Henri Mondor, France

***Correspondence:**
Ingrid van der Mei
ingrid.vandermei@utas.edu.au

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: 10 February 2018
Accepted: 22 May 2018
Published: 08 June 2018

Citation:
Tao C, Simpson S Jr, Taylor BV, Blizzard L, Lucas RM, Ponsonby A-L, Broadley S, AusLong/Ausimmune Investigators Group and van der Mei I (2018) Onset Symptoms, Tobacco Smoking, and Progressive-Onset Phenotype Are Associated With a Delayed Onset of Multiple Sclerosis, and Marijuana Use With an Earlier Onset. Front. Neurol. 9:418. doi: 10.3389/fneur.2018.00418

Background: Age at symptom onset (ASO) is a prognostic factor that could affect the accrual of disability in multiple sclerosis (MS) patients. Some factors are known to influence the risk of multiple sclerosis (MS), but their influence on the ASO is less well-investigated.

Objective: Examine the associations between known or emerging MS risk factors and ASO.

Methods: This was a multicenter study, incident cases ($n = 279$) with first clinical diagnosis of demyelinating event aged 18–59 years recruited at four Australian centres (latitudes 27°-43°S), from 1 November 2003 to 31 December 2006. Environmental/behavioral variables and initial symptoms were recorded at baseline interview. Linear regression was used to assess the association between risk factors and ASO.

Results: Five factors were significantly associated with ASO: a history of tobacco smoking was associated with 3.05-years later ASO ($p = 0.002$); a history of marijuana use was associated with 6.03-years earlier ASO ($p < 0.001$); progressive-onset cases had 5.61-years later ASO ($p = 0.001$); an initial presentation of bowel & bladder and cerebral dysfunctional were associated with 3.39 ($p = 0.017$) and 4.37-years ($p = 0.006$) later ASO, respectively. Other factors, including sex, offspring number, latitude of study site, history of infectious mononucleosis, *HLA-DR15 & HLA-A2* genotype, 25(OH)D levels, and ultraviolet radiation exposure were not associated with ASO. Including all five significant variables into one model explained 12% of the total variance in ASO.

Conclusion: We found a novel association between a history of tobacco smoking and later onset, whereas marijuana use was associated with earlier onset. Behavioral factors seem important drivers of MS onset timing although much of the variance remains unexplained.

Keywords: first demyelinating event, age at symptom onset, smoking, offspring number, marijuana, multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (CNS), caused by a complex interplay between genetic and environmental factors (1). *HLA-DR15*01* genotype (2), lower vitamin D (3) or exposure to ultraviolet radiation (UVR) (4), tobacco use (5), and past infection with Epstein-Barr virus (6) are the main factors implicated in MS risk. Our group recently demonstrated that these risk factors could explain 63.8% of the attributable risk (7), with 53.3% of that due to the environmental factors. Other factors such as *HLA-A2* genotype (8) (protective), offspring number (9) (protective), and marijuana use (5) (detrimental), have some evidence of involvement in MS risk.

While a number of studies have examined risk factors for the onset of MS, far fewer have tested associations with age at symptom onset (ASO), which is the age at which first symptoms suggestive of future MS occur. Current studies (10, 11) mostly suggest a significant prognostic value of ASO in patients with MS, with an early onset correlated with a benign disease progression. Some studies showed that lower sun exposure in adolescence was associated with earlier ASO (12, 13). One cohort study ($n = 895$ cases) found no difference in ASO between never and ever smokers (32.29 and 32.75 years, respectively) (14). One study ($n = 816$) found that cases with *HLA-DR15* risk genotype had roughly 2.5-years earlier onset (15), but a later meta-analysis ($n = 2{,}201$) showed no association (16). The association between *HLA-A2* genotype and ASO has also yielded inconsistent findings (17, 18). Our group (19) found that progressive-onset patients had ~9 years later ASO than relapsing-onset patients ($p < 0.001$).

In view of the conflicting evidence presented above, this paper examines the associations between risk factors for MS onset and ASO in a cohort recruited soon after the first clinical diagnosis of CNS demyelination (FCD).

METHODS

The Ausimmune Study was an Australian multicenter case-control study (20). Case participants were aged 18–59 years and resident in a study region: Brisbane city (latitude 27° South), Newcastle city and surrounds (33°S), Geelong city and the Western Districts of Victoria (37° S), or the state of Tasmania (~41–43°S). Incident cases ($n = 282$) were referred to the study by medical specialists, following a FCD. A study neurologist confirmed the date and symptomatology of the demyelinating event(s) that led to study participation and conducted a full neurologic examination. In subsequent follow-up (AusLong study) (21), 3 cases were confirmed as non-MS, and 19 cases were confirmed as primary-progressive MS (PPMS). Among all 279 cases, 217 had been diagnosed as MS at 5-year review. Among 260 relapsing-onset cases, 219 had their first demyelinating event (FDE) during the study recruitment period (1 Nov 2003 to 31 Dec 2006), with remaining cases having had a prior, previously unrecognized neurological event.

The Ausimmune Study was approved by nine regional Human Research Ethics Committees. All participants gave written informed consent.

Measurements

Demographic, environmental, behavioral and neurological data were collected by self-reported questionnaire and face-to-face interview. Serum samples and biometric measures were taken at face-to-face interview.

Using the Expanded Disability Status Scale (EDSS), clinical presentation was rated for seven function groups (pyramid, cerebellar, brainstem, sensory, bowel & bladder, cerebral, and visual) using 6–8 point scales. It was assessed by a neurologist via face-to-face interview at baseline. Questionnaire data collected included: a detailed smoking history of tobacco and marijuana use (ever smoked, current smoking status, age started/stopped smoking, pack-years of cigarette smoking, pack-years of marijuana use); history of infectious mononucleosis ("have you ever had glandular fever"); number and age of all live births; and for females only, the age at menarche. Participants completed a personal residence calendar (to assist recall of past events), completing for each year of life from age 6 years the location of residence and leisure time in the sun in summer and winter. With the latitude and longitude of the location, average daily ambient erythemally weighted UVR was calculated for every year of life for each participant (4). For each participant, UVR dose (kJ/m^2) was estimated for the relevant periods of life by multiplication of the average hours per day outside in each season by the average UVR for that season, and summed over the relevant years. Annual UVR exposure was assigned by adding the summer and winter UVR dose.

Serum concentrations of 25(OH)D were measured using liquid chromatography tandem mass spectrometry. Skin reflectance on the buttock was measured using a hand-held spectrophotometer (Minolta CM-2500D) to estimate cutaneous melanin density (4). The genotyping of *HLA-DR15* (SNP rs9271366) was performed by the SNPline method (KBiosciences, Hoddesdon Herts, UK). The genotyping of *HLA-A2* (SNP rs2844821 on Illumina Custom MS Chip) was

performed by the Hussman Institute for Human Genomics, University of Miami.

Data Management and Analysis

ASO was calculated by subtracting the date of birth from the date of first symptom onset (as determined by review of data by the study neurologist team). Where only the year of symptom onset was recorded, the onset date was assigned as 15th of June of that year and if only the month was known it was assigned as the 15th of the month [year of FDE was recorded in 27 cases, but the results were robust when these data were omitted from the analysis (data not shown)].

Because 25(OH)D levels vary substantially by season, we deseasonalised 25(OH)D levels in each study center using a sine-cosine function, as described previously (4). The 25(OH)D level was modeled as both a continuous and categorical variable (using commonly used cut-points, <50, 50–75, >75–100, >100 nmol/L).

Due to the non-significant association between the degree of clinical presentation and ASO in patients with each clinical symptom ($p > 0.05$), clinical presentation of the seven function groups were coded as no or yes in our analysis.

Log-binomial regression was used to assess the association between categorical variables. Linear regression was used to assess the association between potential predictors and ASO. We mostly examined risk factors that occurred prior to the initial symptom onset to ensure proper temporality, except 25(OH)D was measured at the baseline review. For variables that were inherently associated with age (e.g., smoking, UVR exposure, offspring number), we summarized the information prior to a specific age (e.g., 28 years for smoking, 31 years for marijuana and offspring number) and examined the associations with ASO after this age to obtain a less biased estimate. The distribution of ASO was left skewed, and a Box-Cox power transformation was applied to reduce heteroskedasticity and thus satisfy the requirements of linear regression. All coefficients presented in the results were back-transformed to the original scale.

In preliminary analyses, participants in Tasmania had a later ASO than other study centers (mean difference: 3.19, 95% CI 0.73–5.65, $p = 0.011$). The higher ASO was likely driven by the higher average age of the Tasmanian population, but the collinearity between the population mean age and study center ($r = 0.91$, $p < 0.001$) made adjustment not possible. We therefore divided cases into five-year ASO groups (15–19 years, 20–24 years, 25–29 years, 30–34 years, 35–39 years, 40–44 years, 45–49 years, 50–54 years, 55–59 years) in each study center, and standardized the ASO to the age distribution in the whole Australian population. After standardization, ASO (from highest to lowest latitude: 37.2, 37.6, 37.0 & 37.2 years, respectively) was similar in the four different study centers.

In the basic multivariable model, we adjusted for sex, study center, and MS onset type (relapsing vs. progressive onset). For UVR exposure analysis, we did not adjust for study center due to collinearity (latitude and ambient UVR are strongly negatively correlated). We adjusted for buttock melanin density to take natural skin type into consideration. For smoking analyses, we further adjusted for tobacco or marijuana smoking, due to the significant positive correlation ($r = 0.40$, $p < 0.001$).

After the univariable and multivariable analyses of each category of exposures, we built a mutually adjusted model including all significant exposures, and examined the total variance explained by these exposures. The relative contribution of each variable was calculated as the change of the residual sum of squares when comparing the model without the variable with the full model.

We conducted a sensitivity analysis restricted to cases with FDE during the recruitment period and excluding those whose ASO was less certain. We performed another separate analysis restricted to cases who had converted to MS by a 5-year follow-up, (21) thus excluding cases who may never convert to MS. We were concerned that some associations were the result of an age or cohort effect. To examine this we used the controls ($n = 545$) and assigned the same ASO of the cases to their age and sex-matched controls. With the Australian Electoral Roll, we randomly selected controls and matched to cases on age (within two years), sex, and study region (60% participated of those originally contacted). The final case:control matching ratio averaged 1:2 (20). If an association was also observed in controls, then that is likely due to an age or cohort effect.

All statistical analyses were undertaken using Stata/SE 12.1 (College Park, TX).

RESULTS

The mean ASO for the total cohort was 37.3 years and 76.7% were females. ASO for those who had converted to MS at 5-year follow-up ($n = 217$) was 37.0 years and for cases with FDE during the recruitment period ($n = 219$) was 36.9 years. All primary analyses were based on all patients with FDE ($n = 279$), and sensitivity analyses including patients had converted to MS at 5-year review ($n = 217$) or patients with FDE during recruitment period ($n = 219$) were presented in the last section of result.

Sex, HLA-DR15, HLA-A2 and History of Infectious Mononucleosis

We found no significant associations between ASO and sex, history of IM, *HLA-DR15* genotype, or *HLA-A2* genotype (**Table 1**; Supplementary Table 1).

25(OH)D and UVR Dose

No seasonal pattern was seen in the association between season of FDE and ASO ($F = 0.61$, $p = 0.72$). There was no association between deseasonalised 25(OH)D level and ASO (**Table 1**). Time between FDE and 25(OH)D being taken did not modify the association between 25(OH)D and ASO ($p_{interactio}$ $n = 0.38$). Cumulative UVR dose (summer, winter, and combined) within different risk windows (e.g., 5, 10, and 15 years) prior to onset and UVR dose during adolescence (6-15 years) showed no convincing associations with ASO (**Table 1**; Supplementary Table 2).

Onset Type and Initial Symptoms

ASO of progressive-onset patients was 5.61-years later than relapsing-onset patients ($p = 0.013$) (**Table 2**). There was

TABLE 1 | Associations between sex, IM history, HLA-DR15 genotype, HLA-A2 genotype, 25(OH)D, UVR exposure and age of symptom onset.

		Univariable		Multivariable	
	No. (%)	**β (95% CI)**	***p***	**β (95% CI)**	***p***
SEX[a]					
Male	65 (23.30)	37.50 (35.17, 39.83) [c]		37.21 (34.90, 39.52)[c]	
Female	214 (76.70)	−0.28 (−2.94, 2.38)	0.84	+0.15 (-2.49, 2.80)	0.91
PAST HISTORY OF IM[a]					
No	200 (72.99)	37.61 (36.30, 38.92)[c]		37.7 (36.41, 39.00)[c]	
Yes	74 (27.01)	−1.18 (−3.70, 1.34)	0.36	−1.33 (−3.83, 1.18)	0.30
PAST HISTORY OF IM (EXCLUDING THOSE WHO DID NOT KNOW WHETHER THEY HAD A HISTORY OF IM)[a]					
No	181 (70.98)	37.23 (35.85, 38.61)[c]		37.32 (35.96, 38.67)[c]	
Yes	74 (29.02)	−0.85 (−3.41, 1.71)	0.51	−0.96 (−3.49, 1.58)	0.46
HLA-DR15 (RS9271366)[a]					
AA	106 (43.80)	37.19 (35.38, 39.00)[c]		36.89 (34.91, 38.86)[c]	
AG	123 (50.83)	+0.40 (−2.07, 2.86)	0.75	+1.42 (−1.26, 4.09)	0.30
GG	13 (5.37)	+3.18 (−2.26, 8.63)	0.25	+4.74 (−2.43, 11.91)	0.20
Trend			0.38		0.15
HLA–A2 (RS2844821)[a]					
AA	117 (54.42)	36.93 (35.22, 38.64)[c]		37.17 (35.43, 38.92)[c]	
AG	98 (45.58)	+1.79 (−0.74, 4.31)	0.17	+1.38 (−1.24, 4.01)	0.30
SEASON OF FDE[b]					
Spring	70 (25.09)	36.9 (34.47, 39.34)[c]		37.2 (34.69, 39.7)[c]	
Summer	72 (25.81)	+1.00 (−2.27, 4.26)	0.55	0.01 (−3.39, 3.42)	0.99
Autumn	61 (21.86)	+0.05 (−3.28, 3.37)	0.98	−0.42 (−3.8, 2.97)	0.81
Winter	76 (27.24)	+0.40 (−2.87, 3.66)	0.81	0.43 (−2.89, 3.76)	0.80
Deseasonalised 25(OH)D, continuous (per 10 nmol/L increase)[b]		−0.04 (−0.48, 0.40)	0.87	−0.03 (−0.48, 0.41)	0.88
DESEASONALISED 25(OH)D[b]					
<50 nmol/L	33 (15.57)	36.95 (33.68, 40.22)[c]		36.92 (33.64, 40.19)[c]	
50–75 nmol/L	70 (33.02)	−0.19 (−4.16, 3.77)	0.92	−0.33 (−4.32, 3.65)	0.87
76 – 100 nmol/L	70 (33.02)	−0.68 (−4.65, 3.28)	0.74	−0.75 (−4.72, 3.22)	0.71
> 100 nmol/L	39 (18.40)	+1.26 (−3.18, 5.71)	0.58	+1.19 (−3.25, 5.64)	0.60
Trend			0.67		0.67
WINTER UVR DOSE IN THE 15 YEARS BEFORE ONSET[b]					
0–0.93 x10^2 kJ/m^2	64 (24.81)	39.62 (37.43, 41.81)[c]		39.68 (37.47, 41.9)[c]	
> 0.93–1.59 × 10^2 kJ/m^2	65 (25.19)	−3.05 (−6.12, 0.02)	0.05	***−3.37 (−6.46, −0.28)***	***0.032***
> 1.59–2.51 × 10^2 kJ/m^2	64 (24.81)	−1.95 (−5.04, 1.14)	0.22	−2.06 (−5.22, 1.09)	0.20
> 2.51–8.47 × 10^2 kJ/m^2	65 (25.19)	−1.43 (−4.51, 1.65)	0.36	−1.37 (−4.50, 1.76)	0.39
Trend			0.53		0.57
SUMMER UVR DOSE IN THE 15 YEARS BEFORE ONSET[b]					
0–5.53 x10^2 kJ/m^2	64 (24.71)	39.82 (37.66, 41.97)[c]		39.54 (37.35, 41.73)[c]	
> 5.53–7.37 × 10^2 kJ/m^2	65 (25.10)	***−3.91 (−6.91, −0.91)***	***0.011***	***−3.75 (−6.77, −0.73)***	***0.015***
> 7.37–9.99 × 10^2 kJ/m^2	65 (25.10)	***−3.95 (−6.95, −0.95)***	***0.010***	***−3.87 (−6.93, −0.81)***	***0.013***
> 9.99–18.99 × 10^2 kJ/m^2	65 (25.10)	+0.38 (−2.66, 3.42)	0.81	+1.07 (−2.15, 4.28)	0.52
Trend			0.81		0.65
ANNUAL UVR DOSE IN THE 15 YEARS BEFORE ONSET[b]					
0–6.87 × 10^2 kJ/m^2	64 (24.81)	39.35 (37.18, 41.52)[c]		39.09 (36.89, 41.3)[c]	
> 6.87–9.30 × 10^2 kJ/m^2	65 (25.19)	***−3.13 (−6.15, −0.10)***	***0.043***	−3.01 (−6.07, 0.04)	0.053
> 9.30–12.00 × 10^2 kJ/m^2	64 (24.81)	***−3.42 (−6.45, −0.38)***	***0.027***	***−3.26 (−6.37, −0.15)***	***0.040***
> 12.00–22.46 × 10^2 kJ/m^2	65 (25.19)	+0.85 (−2.21, 3.91)	0.59	+1.41 (−1.79, 4.61)	0.39
Trend			0.64		0.48

Statistical significance ($p < 0.05$) is denoted in bold and italics.
[a]adjusted for sex, MS onset type and study center; [b]adjusted for sex, MS onset type and buttock melanin density; [c]mean ASO of the reference group.
MS, multiple sclerosis; IM, infectious mononucleosis; UVR, ultraviolet radiation; FDE, first demyelinating event.

TABLE 2 | Associations between onset type and initial symptomatology and age of symptom onset.

		Univariable analysis		Multivariable analysis[a]	
	No. (%)	β (95% CI)	*p*	β (95% CI)	*p*
MS ONSET TYPE					
Relapsing-onset	258 (92.47)	36.99 (35.84, 38.15)[b]		36.95 (35.8, 38.09)[b]	
Progressive-onset	21 (7.53)	***+4.86 (0.44, 9.28)***	***0.031***	***+5.61 (1.17, 10.05)***	***0.013***
ONSET SYMPTOMS					
Pyramidal dysfunction -no	150 (58.37)	37.51 (35.98, 39.04)[b]		37.86 (36.33, 39.38)[b]	
Pyramidal dysfunction -yes	107 (41.63)	+0.21 (−2.17, 2.58)	0.86	−0.52 (−2.94, 1.90)	0.67
Cerebellar dysfunction -no	185 (72.27)	36.89 (35.53, 38.26)[b]		37.01 (35.66, 38.37)[b]	
Cerebellar dysfunction -yes	71 (27.73)	***+2.83 (0.24, 5.41)***	***0.032***	+2.52 (−0.08, 5.12)	0.06
Brainstem dysfunction -no	192 (75.59)	37.43 (36.06, 38.79)[b]		37.4 (36.06, 38.75)[b]	
Brainstem dysfunction -yes	62 (24.41)	−0.13 (−2.90, 2.64)	0.93	0.15 (−2.59, 2.89)	0.92
Sensory dysfunction -no	114 (45.6)	37.6 (35.83, 39.37)[b]		37.56 (35.81, 39.3)[b]	
Sensory dysfunction -yes	136 (54.4)	−0.48 (−2.88, 1.92)	0.70	−0.33 (−2.70, 2.05)	0.79
Bowel & Bladder dysfunction -no	210 (79.55)	36.84 (35.56, 38.12)[b]		36.85 (35.58, 38.12)[b]	
Bowel & Bladder dysfunction -yes	54 (20.45)	***+3.39 (0.57, 6.20)***	***0.019***	***+3.49 (0.63, 6.34)***	***0.017***
Cerebral dysfunction -no	218 (83.52)	36.71 (35.47, 37.95)[b]		36.87 (35.64, 38.1)[b]	
Cerebral dysfunction-yes	43 (16.48)	***+5.12 (2.08, 8.15)***	***0.001***	***+4.37 (1.28, 7.46)***	***0.006***
Visual dysfunction -no	178 (70.92)	37.64 (36.25, 39.03)[b]		37.74 (36.38, 39.10)[b]	
Visual dysfunction -yes	73 (29.08)	−0.80 (−3.38, 1.77)	0.54	−1.00 (−3.54, 1.54)	0.44
ONSET SYMPTOMS IN RELAPSING-ONSET CASES					
Pyramidal dysfunction -no	147 (61.51)	37.22 (35.70, 38.75)[b]		37.35 (35.84, 38.86)[b]	
Pyramidal dysfunction -yes	92 (38.49)	−0.09 (−2.55, 2.37)	0.94	−0.36 (−2.80, 2.09)	0.77
Cerebellar dysfunction -no	179 (74.27)	36.55 (35.18, 37.92)[b]		36.55 (35.19, 37.91)[b]	
Cerebellar dysfunction -yes	62 (25.73)	***+2.82 (0.11, 5.52)***	***0.041***	***+2.91 (0.21, 5.60)***	***0.034***
Brainstem dysfunction -no	179 (74.9)	36.79 (35.39, 38.18)[b]		36.78 (35.41, 38.16)[b]	
Brainstem dysfunction -yes	60 (25.1)	+1.06 (−1.72, 3.83)	0.46	+1.14 (−1.61, 3.90)	0.42
Sensory dysfunction -no	106 (45.3)	37.15 (35.34, 38.96)[b]		37.12 (35.33, 38.91)[b]	
Sensory dysfunction -yes	128 (54.7)	−0.29 (−2.73, 2.15)	0.81	−0.21 (−2.63, 2.21)	0.87
Bowel & Bladder dysfunction -no	201 (81.71)	36.52 (35.23, 37.81)[b]		36.43 (35.16, 37.71)[b]	
Bowel & Bladder dysfunction -yes	45 (18.29)	***+3.33 (0.31, 6.34)***	***0.031***	***+3.90 (0.88, 6.93)***	***0.011***
Cerebral dysfunction -no	205 (84.36)	36.35 (35.10, 37.61)[b]		36.47 (35.21, 37.72)[b]	
Cerebral dysfunction -yes	38 (15.64)	***+5.20 (2.02, 8.39)***	***0.001***	***+4.54 (1.30, 7.78)***	***0.006***
Visual dysfunction -no	166 (70.34)	37.10 (35.68, 38.51)[b]		37.23 (35.83, 38.63)[b]	
Visual dysfunction -yes	70 (29.66)	−0.24 (−2.84, 2.37)	0.86	−0.63 (−3.22, 1.96)	0.63

Statistical significance ($p < 0.05$) is denoted in bold and italics. [a]adjusted for sex, MS type, and study center; [b]mean ASO of the reference group.

no association between pyramidal, brainstem, sensory, visual dysfunction and ASO. Cases with bowel/bladder and cerebral symptoms had 3.49-years ($p = 0.017$) and 4.37-years ($p = 0.006$) later onset, respectively, persisting on restriction to relapsing-onset cases. Patients with cerebellar function impairment had a 2.52-years later onset than those without cerebellar function impairment ($p = 0.057$), and this association became significant when restricting to relapsing-onset cases.

Smoking of Tobacco and Marijuana

Ever smokers had ∼5.11-years later onset than never smokers ($p < 0.001$) (**Table 3**). The magnitudes were similar between past smokers and current smokers. Cases with an earlier ASO might have had less opportunity to start smoking, so we tested this by only including cases with ASO ≥ 28 years (as 27 was the oldest age of taking up smoking). While attenuating the magnitude, a history of smoking remained significantly associated with ASO (**Table 3**). We repeated the analysis in the matched controls, and no difference of ASO was shown (never smokers vs. ever smokers: adjusted β = +0.11 years, 95% CI −1.54–1.77, $p = 0.89$), suggesting the significant association in cases was not due to an age or cohort effect. Among ever smokers, the ASO was similar whether the smoking was taken up early (<16 years) or later ($p_{Interactio}$ $n = 0.26$), or the duration was longer or shorter ($p_{Interactio}$ $n = 0.94$). Among cases with ASO ≥ 28 years, total pack years of tobacco use before age 27 was not associated with ASO.

Marijuana use was more common in male cases than in females (OR = 0.50, $p = 0.020$), and a history of marijuana use was positively associated with smoking (OR = 8.05, $p < 0.001$). A

TABLE 3 | Associations between smoking behaviors and age of symptom onset.

		Univariable		Multivariable[a]	
	No. (%)	**β (95% CI)**	***P***	**β (95% CI)**	***P***
SMOKING EVER					
No	103 (37.59)	35.6 (33.79, 37.41)[b]		34.24 (32.41, 36.06)[b]	
Yes	171 (62.41)	***+2.71 (0.42, 5.00)***	***0.021***	***+5.11 (2.74, 7.48)***	***<0.001***
SMOKING EVER (ASO ≥ 28 YEARS)					
No	79 (35.59)	38.71 (37.2, 40.22)[b]		37.89 (36.45, 39.34)[b]	
Yes	143 (64.41)	+1.79 (−0.12, 3.71)	0.07	***+3.05 (1.16, 4.95)***	***0.002***
SMOKING STATUS					
Never smoked	103 (37.59)	35.6 (33.78, 37.41)[b]		34.21 (32.38, 36.03)[b]	
Past smokers	101 (36.86)	***+2.62 (0.04, 5.20)***	***0.047***	+4.80 (2.21, 7.38)	***<0.001***
Current smokers	70 (25.55)	+2.85 (−0.01, 5.70)	0.05	+5.66 (2.69, 8.64)	***<0.001***
Trend			***0.037***		***<0.001***
SMOKING STATUS (ASO ≥ 28 YEARS)					
Never smoked	79 (35.59)	38.71 (37.2, 40.22)[b]		37.89 (36.44, 39.34)[b]	
Past smokers	83 (37.39)	+2.12 (−0.05, 4.30)	0.06	***+3.05 (0.95, 5.14)***	***0.004***
Current smokers	60 (27.03)	+1.34 (−1.01, 3.68)	0.27	***+3.06 (0.65, 5.47)***	***0.013***
Trend			0.22		***0.010***
AGE UPTAKE SMOKING (ASO ≥ 28 YEARS)					
≥16 years	84 (58.74)	41.45 (39.85, 43.05)[b]		41.21 (39.72, 42.7)[b]	
<16 years	59 (41.26)	−1.77 (−4.22, 0.68)	0.16	−1.35 (−3.66, 0.97)	0.26
DURATION OF SMOKING BEFORE AGE 27 (ASO ≥ 28 YEARS)					
≤10 years	55 (40.44)	40.97 (38.99, 42.95)[b]		40.58 (38.67, 42.5)[b]	
>10 years	81 (59.56)	−0.51 (−3.07, 2.04)	0.69	+0.10 (−2.40, 2.60)	0.94
TOTAL PACK YEARS OF SMOKING BEFORE AGE 27 (ASO ≥ 28 YEARS)					
0	93 (41.33)	39.19 (37.77, 40.61)		39.10 (37.71, 40.49)	
1–2000	58 (25.78)	+0.50 (−1.81, 2.81)	0.67	+0.61 (−1.67, 2.89)	0.60
>2000–20000	74 (32.89)	+2.05 (−0.15, 4.25)	0.07	+2.24 (0.07, 4.41)	0.043
Trend			0.07		0.048
MARIJUANA EVER					
No	190 (69.6)	38.58 (37.26, 39.9)[b]		39.23 (37.91, 40.55)[b]	
Yes	83 (30.4)	***−4.04 (−6.43, −1.64)***	***0.001***	−6.03 (−8.62, −3.45)	***<0.001***
MARIJUANA EVER (ASO ≥ 31 YEARS)					
No	151 (74.02)	41.29 (40.24, 42.34)[b]		41.5 (40.43, 42.57)[b]	
Yes	53 (25.98)	***−2.20 (−4.14, −0.27)***	***0.026***	***−2.80 (−4.89, −0.71)***	***0.009***
MARIJUANA STATUS					
Never	190 (69.6)	38.58 (37.26, 39.90)[b]		38.56 (37.25, 39.87)[b]	
Past users	63 (23.08)	***−4.08 (−6.73, −1.43)***	***0.003***	***−3.98 (−6.63, −1.34)***	***0.003***
Current users	20 (7.33)	−3.92 (−8.20, 0.37)	0.07	−3.46 (−7.87, 0.94)	0.12
Trend			***0.003***		***0.004***
MARIJUANA STATUS (ASO ≥ 31 YEARS)					
Never	151 (74.02)	41.28 (40.24, 42.33)[b]		41.25 (40.22, 42.28)[b]	
Past users	39 (19.12)	−1.50 (−3.71, 0.70)	0.18	−1.35 (−3.58, 0.88)	0.24
Current users	14 (6.86)	***−4.06 (−7.16, −0.95)***	***0.010***	***−3.88 (−7.09, −0.66)***	***0.018***
Trend			***0.008***		***0.016***
TOTAL PACK YEARS OF MARIJUANA USE BEFORE AGE 30 (ASO ≥ 31 YEARS)					
0	180 (86.96)	40.96 (40.01, 41.92)		40.93 (39.99, 41.87)	
1–30	13 (6.28)	+1.13 (−2.70, 4.96)	0.56	+1.48 (−2.34, 5.30)	0.45
>30	14 (6.76)	−3.16 (−6.34, 0.02)	0.052	−3.07 (−6.29, 0.14)	0.06
Trend			0.14		0.19

Statistical significance ($p < 0.05$) was denoted in bold and italics. [a]adjusted for sex, MS type, study centers and tobacco smoking status/marijuana use status; [b]mean ASO of the reference group.

history of marijuana use was associated with a 6.03-years earlier ASO (**Table 3**). The magnitudes were similar between past users and current users. We next evaluated associations among cases with ASO ≥ 31 years (30 years was the oldest age of taking up marijuana in this cohort). Magnitudes were attenuated but still significant in this subgroup (**Table 3**). Among cases with ASO ≥ 31 years, total pack years of marijuana use before age 30 was not associated with ASO.

Offspring Numbers and Age at Menarche

Having children was strongly associated with a later ASO (**Table 4**). The magnitude was potentially biased since those with a later ASO may have had different opportunities to have children. We therefore chose a cut-off point that balanced sample size against the offspring number information, and by restricting to those with ASO ≥ 31 years, we could include 204 cases and 56.9% of participant offspring. A significant dose-dependent association remained, albeit with reduced magnitude. This dose-dependent association was found in both male and female participants ($p_{\text{interactio}}\ n = 0.11$). When we repeated the analyses in the controls, having more children was also significantly associated with a later ASO (0 vs. 1: $\beta = 4.55$, $p < 0.001$; 0 vs. ≥2: $\beta = 10.41$, $p < 0.001$; $p_{\text{trend}} < 0.001$), and restricting to those with ASO ≥ 31 years still showed a significant association (0 vs. 1: $\beta = 1.39$, $p = 0.16$; 0 vs. ≥ 2: $\beta = 3.48$, $p < 0.001$; $p_{\text{trend}} < 0.001$). This suggests that the significant association of offspring number and ASO in cases was largely driven by an age or cohort effect.

The age of the participant when having their first child (≤20, 21–25, 26–30, 31–35, ≥36) was not associated with ASO; the average ASO was around 41 years for each child birth age group. There was no association between age of menarche and ASO ($p = 0.27$).

Mutually Adjusted Model

We next built a combined model based on the significant findings (tobacco smoking, marijuana use, MS onset type, cerebral dysfunction, and bowel & bladder dysfunction). The final model explained ∼12% of the total variance in ASO, with marijuana use and tobacco smoking having the largest relative contributions (**Table 5**).

Sensitivity Analyses

Sensitivity analyses that restricted to cases with FDE during the study recruitment period or diagnosed MS cases did not materially alter the results (Supplementary Tables).

DISCUSSION

In this comprehensive study, we systematically evaluated the association between ASO and risk factors for MS onset. A model including all the significant factors explained 12% of the total variance. Of the individual risk factors, a history of tobacco smoking, progressive-onset type, and cerebral or bowel and bladder symptoms at FDE were associated with a later onset, while marijuana use was associated with an earlier onset. *HLA-A2* genotype, *HLA-DR15* genotype, latitude of study site, past UVR dose, current 25(OH)D levels, history of IM, offspring number, age of menarche, and sex were not associated with ASO.

A strength of our study is the detailed information on exposure variables, as well as the study neurologist interview and review of medical records for optimal assessment of the ASO. Also, our findings showed a robustness when repeating the analysis among those diagnosed with MS by 5-year review (excluding some cases who may not convert to MS), as well as restricting to cases with FDE during the study recruitment

TABLE 4 | Associations between offspring numbers/age at menarche and age of symptom onset.

		Univariable		Multivariable	
	No. (%)	**β (95% CI)**	***p***	**β (95% CI)**	***p***
OFFSPRING NUMBER[a]					
0	111 (39.78)	31.16 (29.7, 32.63)[c]		31.37 (29.9, 32.83)[c]	
1	39 (13.98)	***+5.97 (3.01, 8.92)***	***<0.001***	***+5.51 (2.57, 8.44)***	***<0.001***
2 or more	129 (46.24)	***+11.12 (9.06, 13.18)***	***<0.001***	***+10.88 (8.84, 12.93)***	***<0.001***
Trend			***<0.001***		***<0.001***
OFFSPRING NUMBER (ASO ≥ 31 YEARS)[b]					
0	89 (43.00)	39.25 (37.99, 40.5)[c]		39.22 (37.99, 40.45)[c]	
1	37 (17.87)	+1.67 (−0.74, 4.09)	0.18	+1.39 (−0.98, 3.77)	0.25
2 or more	81 (39.13)	***+3.42 (1.47, 5.36)***	***0.001***	***+3.61 (1.69, 5.54)***	***<0.001***
Trend			***0.001***		***<0.001***
AGE OF MENARCHE[a]					
8–12 years	98 (46.23)	37.07 (35.17, 38.97)[c]		37.13 (35.24, 39.01)[c]	
13–14 years	87 (41.04)	+1.13 (−1.64, 3.9)	0.43	+0.98 (−1.8, 3.75)	0.49
15–23 years	27 (12.74)	***−4.19 (−8.24, −0.15)***	***0.042***	−3.72 (−7.76, 0.32)	0.07
Trend			0.22		0.27

Statistical significance (p<0.05) is denoted in bold and italics. [a]Models adjusted for sex, MS type, and study centre; [b]samples limited to those with ASO ≥ age 31 years and summarizing offspring number < age 31 years, models adjusted for sex, MS type, and study centers. [c]mean ASO of the reference group.

TABLE 5 | Mutually adjusted model including all factors associated with age of symptom onset.

	All cases (adjusted $R^2 = 0.12$)		
	β (95% CI)	*P*	RC
Smoking status			5.0%
Never smoking	35.14 (33.31, 36.98)[a]		
Past users	+3.80 (1.21, 6.40)	***0.004***	
Current users	+4.72 (1.74, 7.71)	***0.002***	
Trend		***0.001***	
Marijuana history			9.2%
Never marijuana	39.47 (38.17, 40.78)[a]		
Past users	−5.49 (−8.29, −2.70)	***<0.001***	
Current users	−7.23 (−11.75, −2.71)	***0.002***	
Trend		***<0.001***	
MS onset type			0.6%
RRMS	37.58 (36.46, 38.69)[a]		
PPMS	+2.23 (−2.05, 6.52)	0.31	
Cerebral function			5.9%
No	36.96 (35.78, 38.14)[a]		
Yes	+4.58 (1.68, 7.48)	***0.002***	
Bowel & bladder function			1.2%
No	37.3 (36.09, 38.51)[a]		
Yes	+2.13 (−0.61, 4.87)	0.13	

Statistical significance ($p < 0.05$) is denoted in bold and italics. RC, relative contribution.
[a]mean ASO of the reference group.

period (excluding some whose ASO was slightly less certain). A challenge was that some exposures were intrinsically associated with ASO simply as a function of time. So we assessed the temporal relationship through analyzing some exposure variables before some age points and analyzing the association with ASO after this point. This ensured that the participants included in the analysis had an equal opportunity of exposure, but it substantially reduced the power and assumed that there was nothing unusual about those with a later vs. earlier onset. To examine whether some observed associations were due to an age or cohort effect, we tested whether an effect existed in the age and sex matched controls. Based on that analysis, we believe that the association between offspring number and ASO was not a true association.

In agreement with previous studies (19, 22, 23), progressive-onset patients showed a later ASO than relapsing-onset patients. In this study, patients with cerebral dysfunction (related to cognition and mood) or bowel and bladder symptoms at FDE had a 4.4 and 3.5 years later ASO, respectively, and cerebellar function impairment (related to ataxia and coordination) was significantly associated with a later onset in relapse-onset patients (2.91 years later ASO). A previous study (24) found no association between psychiatric ($p = 0.31$) or cognitive ($p = 0.93$) symptoms and ASO, but a significant relationship between sphincteric symptoms and ASO ($p = 0.012$). Marked differences in the ASO between that study and presented study (~30 years vs. 37 years) may account for the divergent findings.

The finding that tobacco smoking delayed the average ASO by 4 years is counterintuitive given that it is a risk factor for MS (5). Two other studies have shown a similar directions of effect, with magnitudes of 2.60 years ($n = 7{,}883$) (25) and 0.82 years ($n = 540$) (13). A null association between tobacco smoking and ASO in healthy controls supported that this association was not due to an age or cohort effect. However, we note that our controls did not show the expected decline in ever smoker status by birth cohort 2004–05 as the general Australian population (26). Some hidden confounder or responder bias, e.g., with older controls being more health conscious and less likely to be smokers may account for this (26). However, the control sample was still a preferable comparison source than the general Australian population, since controls resided in the same regional locations as the cases and were age/sex-matched. The participation rate for controls was lower than cases (20) but this potential volunteer bias would be unlikely to lead to altered patterns of smoking by age among the controls only. A good explanation for the opposite relationship for tobacco use in MS onset (27) versus ASO (13, 25) is challenging. It could relate to the anti-inflammatory effects of nicotine upon T-cells, B-cells, and even dendritic cells (28). Tobacco smoking may have a greater impact on neurodegeneration rather than inflammation and immune dysfunction (29) and older onset cases may have a greater neurodegenerative component (30).

Marijuana use was associated with a 6.0-years earlier ASO, despite the Ausimmune Study (5) showing no association between marijuana use and MS risk [never vs. ever, adjusted OR 0.91 (0.84–1.54)]. Other research has demonstrated a detrimental effect of marijuana on the CNS. Two studies (31, 32) have showed that marijuana use was associated with poorer cognitive performance in MS patients. Thus, there is some evidence supporting potential deleterious impacts of marijuana use in MS, though the means by which this occurs, requires further study.

Consistent with previous research (12, 13, 17, 33), we found no association between sex, history of infectious mononucleosis, *HLA-A2* or *HLA-DR15* genotype and ASO. Latitude of study site, deseasonalised 25(OH)D levels and UVR dose were not associated with ASO in the present study. Using the large MSBase global dataset ($n = 22{,}162$), we previously showed an inverse association between latitude and ASO (every 10° increase in latitude was associated with a 0.82-year earlier onset, $p = 2.20 \times 10^{-13}$) (19). However, in line with the present study, subgroup analysis restricting to Australian participants showed a non-significant association. Our previous study also found that UVR exposure from age 6 to 15 years and lifetime actinic damage were not associated with ASO (34). Another cross-sectional study (12) ($n = 1{,}161$) found that vitamin D-associated SNPs and vitamin D supplementation (multivitamin/vitamin D or fatty fish) were not associated with ASO.

While we identified some factors that were associated with ASO, we explained only 12% of the total variance, thus leaving the majority unexplained. We demonstrated that marijuana use was associated with an earlier MS onset and that a history of tobacco smoking was associated with a later ASO. Further research is required to better understand these opposite effects and underlying pathways. These results, if corroborated and

supported in other studies, may aid in the understanding of MS and potentially contribute to delaying MS onset.

AUTHOR CONTRIBUTIONS

CT analyzed the data, wrote the draft manuscript and completed revisions. IvdM designed this study and formulated the hypotheses for this analysis. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and read and approved the final manuscript.

FUNDING

The work was supported by the National Multiple Sclerosis Society of the United States of America (Award RG3364A1/2), the National Health and Medical Research Council of Australia (APP316901 and 224215), the Australian Research Council, The Royal Australasian College of Physicians, the ANZ William Buckland Foundation, Multiple Sclerosis Research Australia and Bayer Schering Pharma and Biogen Idec. Funding sources did not contribute to the design and conduct of the study, management, analysis or interpretation of the data or approval of the manuscript.

AUSIMMUNE/AUSLONG INVESTIGATORS GROUP

RL (National Centre for Epidemiology and Population Health, Canberra), Keith Dear (Duke Kunshan University, Kunshan, China), A-LP and Terry Dwyer (Murdoch Childrens Research Institute, Melbourne, Australia), IvdM, LB, SS, and BT (Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia), SB (School of Medicine, Griffith University, Gold Coast Campus, Australia), Trevor Kilpatrick (Centre for Neurosciences, Department of Anatomy and Neuroscience, University of Melbourne, Melbourne, Australia). David Williams and Jeanette Lechner-Scott (University of Newcastle, Newcastle, Australia), Cameron Shaw and Caron Chapman (Barwon Health, Geelong, Australia), Alan Coulthard (University of Queensland, Brisbane, Australia), Michael P Pender (The University of Queensland, Brisbane, Australia) and Patricia Valery (QIMR Berghofer Medical Research Institute, Brisbane, Australia).

ACKNOWLEDGMENTS

We express our heartfelt thanks to the participants in the Ausimmune & AusLong studies for their time and energy, without which we could not have realized this work.

The authors also thank the paid research personnel, including the local research officers: Susan Agland, BN, Hunter New England Health, Newcastle, New South Wales; Barbara Alexander, BN, Queensland Institute for Medical Research, Queensland; Marcia Davis, MD, Queensland Institute for Medical Research, Queensland; Zoe Dunlop, BN, Barwon Health, Geelong Hospital, Victoria; Rosalie Scott, BN, Royal Brisbane and Women's Hospital, Queensland; Marie Steele, RN, Royal Brisbane and Women's Hospital, Queensland; Catherine Turner, MPH&TM, Menzies Research Institute, Tasmania; Brenda Wood, RN, Menzies Research Institute, Tasmania; and the Ausimmune Study project officers during the course of the study: Jane Gresham, MA (Int Law), National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory; Camilla Jozwick, BSc(Hons), National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory; Helen Rodgers, RN, National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00418/full#supplementary-material

REFERENCES

1. Mechelli R, Annibali V, Ristori G, Vittori D, Coarelli G, Salvetti M. Multiple sclerosis etiology: beyond genes and environment. *Exp Rev Clin Immunol.* (2010) **6**:481–90. doi: 10.1586/eci.10.11
2. Haines JL, Terwedow HA, Burgess K, Pericak-Vance MA, Rimmler JB, Martin ER, et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The multiple sclerosis genetics group. *Hum Mol Genet.* (1998) **7**:1229–34. doi: 10.1093/hmg/7.8.1229
3. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* (2006) **296**:2832–8. doi: 10.1001/jama.296.23.2832
4. Lucas RM, Ponsonby AL, Dear K, Valery PC, Pender MP, Taylor BV, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* (2011) **76**:540–8. doi: 10.1212/WNL.0b013e31820af93d
5. Ponsonby AL, Lucas RM, Dear K, van der Mei I, Taylor B, Chapman C, et al. The physical anthropometry, lifestyle habits and blood pressure of people presenting with a first clinical demyelinating event compared to controls: the Ausimmune study. *Mult Scler.* (2013) **19**:1717–25. doi: 10.1177/1352458513483887
6. Tao C, Simpson S Jr, Taylor BV, van der Mei I. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. *J Neurol Sci.* (2017) **372**:239–49. doi: 10.1016/j.jns.2016.11.060
7. van der Mei I, Lucas RM, Taylor BV, , Valery PC, Dwyer T, Kilpatrick TJ, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. *Mult Scler.* (2016) **22**:461–9. doi: 10.1177/1352458515594040
8. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* (2011) **476**:214–9. doi: 10.1038/nature10251
9. Ponsonby AL, Lucas RM, van der Mei IA, Dear K, Valery PC, Pender MP, et al. Offspring number, pregnancy, and risk of a first clinical demyelinating event: the AusImmune Study. *Neurology.* (2012) **78**:867–74. doi: 10.1212/WNL.0b013e31824c4648

10. Cierny D, Lehotsky J, Hanysova S, Michalik J, Kantorova E, Sivak S, et al. The age at onset in Multiple Sclerosis is associated with patient's prognosis. *Bratisl Lek Listy* (2017) **118**:374–7. doi: 10.4149/BLL_2017_071
11. Ramachandran S, Strange RC, Jones PW, Kalra S, Nayak D and Hawkins CP. Associations between onset age and disability in multiple sclerosis patients studied using MSSS and a progression model. *Mult Scler Relat Disord.* (2014) **3**:593–9. doi: 10.1016/j.msard.2014.06.002
12. Laursen JH, Sondergaard HB, Sorensen PS, Sellebjerg F and Oturai AB. Association between age at onset of multiple sclerosis and vitamin D level-related factors. *Neurology* (2016) **86**:88–93. doi: 10.1212/WNL.0000000000002075
13. McDowell TY, Amr S, Culpepper WJ, Langenberg P, Royal W, Bever C, et al. Sun exposure, vitamin D and age at disease onset in relapsing multiple sclerosis. *Neuroepidemiology* (2011) **36**:39–45. doi: 10.1159/000322512
14. Manouchehrinia A, Tench CR, Maxted J, Bibani RH, Britton J, Constantinescu CS. Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain* (2013) **136**:2298–304. doi: 10.1093/brain/awt139
15. Masterman T, Ligers A, Olsson T, Andersson M, Olerup O and Hillert J. HLA-DR15 is associated with lower age at onset in multiple sclerosis. *Ann Neurol.* (2000) **48**:211–9. doi: 10.1002/1531-8249(200008)48:2<211::AID-ANA11>3.0.CO;2-R
16. Barcellos LF, Sawcer S, Ramsay PP, Baranzini SE, Thomson G, Briggs F, et al. Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. *Hum Mol Genet.* (2006) **15**:2813–24. doi: 10.1093/hmg/ddl223
17. Smestad C, Brynedal B, Jonasdottir G, Lorentzen AR, Masterman T, Åkesson E, et al. The impact of HLA-A and -DRB1 on age at onset, disease course and severity in Scandinavian multiple sclerosis patients. *Eur J Neurol.* (2007) **14**:835–40. doi: 10.1111/j.1468-1331.2007.01825.x
18. Moutsianas L, Jostins L, Beecham AH, 2, Dilthey AT, Xifara DK, Ban M, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nat Genet.* (2015) **47**:1107–13. doi: 10.1038/ng.3395
19. Tao C, Simpson S Jr., van der Mei I, Blizzard L, Havrdova E, Horakova D, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. *J Neurol Neurosurg Psychiatry* (2016) **87**:1343–9. doi: 10.1136/jnnp-2016-314013
20. Lucas R, Ponsonby AL, McMichael A, van der Mei I, Chapman C, Coulthard A, et al. Observational analytic studies in multiple sclerosis: controlling bias through study design and conduct. The Australian multicentre study of environment and immune function. *Mult Scler.* (2007) **13**:827–39. doi: 10.1177/1352458507077174
21. Pan G, Simpson S Jr, van der Mei I, Charlesworth JC, Lucas R, Ponsonby A-L, et al. Role of genetic susceptibility variants in predicting clinical course in multiple sclerosis: a cohort study. *J Neurol Neurosurg Psychiatry* (2016) **87**:1204–11. doi: 10.1136/jnnp-2016-313722
22. Stankoff B, Mrejen S, Tourbah A, Fontaine B, Lyon-Caen O, Lubetzki C, et al. Age at onset determines the occurrence of the progressive phase of multiple sclerosis. *Neurology* (2007) **68**:779–81. doi: 10.1212/01.wnl.0000256732.36565.4a
23. Confavreux C and Vukusic S. Natural history of multiple sclerosis: a unifying concept. *Brain* (2006) **129**:606–16. doi: 10.1093/brain/awl007
24. Cossburn M, Ingram G, Hirst C, Ben-Shlomo Y, Pickersgill TP and Robertson NP. Age at onset as a determinant of presenting phenotype and initial relapse recovery in multiple sclerosis. *Mult Scler.* (2012) **18**:45–54. doi: 10.1177/1352458511417479
25. Hedstrom AK, Hillert J, Olsson T and Alfredsson L. Smoking and multiple sclerosis susceptibility. *Eur J Epidemiol.* (2013) **28**:867–74. doi: 10.1007/s10654-013-9853-4
26. NHS 2004-05. Available online at: http://www.abs.gov.au/ausstats/abs@.nsf/mf/4831.0.55.001 (Accessed 2016-2017).
27. Hedstrom AK, Hillert J, Olsson T, Alfredsson L. Nicotine might have a protective effect in the etiology of multiple sclerosis. *Mult Scler.* (2013) **19**:1009–13. doi: 10.1177/1352458512471879
28. Filippini P, Cesario A, Fini M, Locatelli F, Rutella S. The Yin and Yang of non-neuronal alpha7-nicotinic receptors in inflammation and autoimmunity. *Curr Drug Targets* (2012) **13**:644–55. doi: 10.2174/138945012800399008
29. Pittas F, Ponsonby AL, van der Mei IA, et al. Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis. *J Neurol.* (2009) **256**:577–85. doi: 10.1007/s00415-009-0120-2
30. Leray E, Yaouanq J, Le Page E, Coustans M, Laplaud D, Oger J, et al. Evidence for a two-stage disability progression in multiple sclerosis. *Brain* (2010) **133**:1900–13. doi: 10.1093/brain/awq076
31. Honarmand K, Tierney MC, O'Connor P, Feinstein A. Effects of cannabis on cognitive function in patients with multiple sclerosis. *Neurology* (2011) **76**:1153–60. doi: 10.1212/WNL.0b013e318212ab0c
32. Pavisian B, MacIntosh BJ, Szilagyi G, Staines RW, O'Connor P, Feinstein A. Effects of cannabis on cognition in patients with MS: a psychometric and MRI study. *Neurology* (2014) **82**:1879–87. doi: 10.1212/WNL.0000000000000446
33. Wu JS, Qiu W, Castley A, Mastaglia FL, Christiansen FT, Christiansen WM, et al. Modifying effects of HLA-DRB1 allele interactions on age at onset of multiple sclerosis in Western Australia. *Mult Scler.* (2010) **16**:15–20. doi: 10.1177/1352458509350312
34. van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Simmons R, Taylor BV, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *BMJ* (2003) **327**:316. doi: 10.1136/bmj.327.7410.316

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 6: Associations between immune responses to Epstein-Barr virus and Human Herpes Virus 6 and multiple sclerosis clinical course

6.1 Preface

In this chapter, we will examine aim 3 of thesis - whether EBV/HHV6 infections could influence conversion to MS, time to relapse and disability with a prospective cohort of patients diagnosed as first clinical diagnosis of CNS demyelination.

6.2 Abstract

Background

Infections with Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV6) have been implicated in multiple sclerosis (MS) onset. Few studies have evaluated the role of these viruses in MS clinical course.

Objective

We evaluated associations between markers of EBV and HHV6 infection and the hazard of subsequent conversion to MS, relapses and annualised change of Expanded Disability Status Scale (EDSS) following a first clinical diagnosis of demyelination (FCD).

Method

The study included 279 cases, followed prospectively for approximately 5 years post FCD. Markers of Epstein-Barr virus and Human Herpes Virus 6 and history of infectious mononucleosis (IM) were measured at the initial interview. Conversion to MS and relapses were assessed at annual reviews and via medical record review, and EDSS was assessed at 5-year review.

Results

Elevated levels of anti-EAR (early antigen, restricted) IgG to EBV was associated with increased hazard of relapses (p=0.023). However, no associations were seen for other serological or viral load parameters of EBV exposure with any aspect of clinical course. History of IM was associated with an increased hazard of relapse (aHR 1.52, 95% CI: 1.16-2.00; p=0.003); however, no association with conversion to MS was seen (p=0.54). Higher levels of anti-HHV6 IgG were associated with an increased annualised change in EDSS (p=0.044). No other associations were seen between HHV6 DNA, anti-HHV6 IgG or anti-HHV6 IgM and any clinical course outcome.

Conclusion

A history of IM and higher levels of anti-EAR IgG at baseline were associated with an increased hazard of relapse, and anti-HHV6 IgG was positively associated with disability progression. This is agreement with other evidence showing a lack of consistent associations between markers of *active* EBV/HHV6 infection and MS clinical course, and inconclusive results in relation to markers of latent infection.

6.3 Introduction

Multiple sclerosis (MS) is a complex autoimmune/neurodegenerative disorder of the central nervous system (CNS). The onset of MS has been ascribed to an interplay between environmental and genetic risk factors[1 2]. Amongst environmental risk factors, the role of infection with human herpesviruses, especially Epstein-Barr virus (EBV), is prominent. Recent meta-analyses support causal relationships between latent EBV infections and increased risk of developing MS, as MS patients showed a higher sero-positivity of immune response to Epstein-Barr nuclear antigen (EBNA, OR=4.5; p<0.001)[3], viral capsid antigen (VCA, OR=4.5; p<0.001)[3] and a past history of infectious mononucleosis (IM, OR=2.17; p<0.001)[4]. However, markers of active infection (increased EBV viral load (EBV-VL) or anti-early antigen (EA)) have not

been consistently associated with increased MS risk[3 5]. Human herpesvirus 6 (HHV6) infections were more prevalent in MS patients, although the evidence is less consistent than that seen with EBV, with some studies showing that anti-HHV6 IgG titres were higher in MS patients than matched controls[6], while others showed no significant differences[7 8].

The issue of whether these viruses are involved in the clinical course of MS - including conversion from clinically isolated syndrome (CIS) to MS, and relapse and disability accumulation following MS diagnosis – is of considerable interest for possible early therapeutic intervention. A recent study[9] involving 468 patients with clinically isolated syndrome (CIS) who were followed for 5 years, found no association between anti-EBV antibodies both baseline and serial and conversion to MS. However, some significant limitations in this study may influence the generalisability of the findings to the whole MS population: initial design of this study was RCT andall patients in this study were accepted interferon-beta. This is consistent with our 2.2 years of follow-up of 198 established MS cases in Tasmania[10], where there was no association between anti-EBNA IgG titres and subsequent risk of relapse. Another prospective study, which followed 147 CIS patients for 7 years, found that a higher anti-EBNA IgG titre at baseline was not associated with the hazard of conversion to MS (p=0.13).[11] However, other studies have shown an association between higher anti-EBNA1 IgG & anti-VCA IgGtitres and increased number of lesions on MRI and/or greater brain atrophy.[12-15]Other studies have demonstrated associations between higher anti-EBNA1 IgG and anti-VCA IgG titres and greater progression of disability, as measured by change in the Expanded Disability Status Scale (EDSS).[11 12 16]

Few studies have examined the association between markers of HHV6 infection and MS clinical course. Following 198 patients with MS for approximate 2 years, our group previously demonstrated a robust dose-dependent positive association between anti-HHV6 IgG levels and subsequent hazard of relapse (p=0.003)[10], while another prospective study[17]including 301 MS patients found that anti-HHV6 IgG titres peaked around two weeks prior to clinical relapse (p=0.01). Higher load of HHV6 DNA in

cerebrospinal fluid (CSF) has been linked with a greater number of contrast-enhancing lesions on MRI[18], in a small study of 37 MS patients.

Here we examined the relationship between markers of EBV and HHV6 infection and clinical course (conversion to MS, relapse and change in disability) in early MS, using a multicentre longitudinal prospective cohort recruited soon after onset of CNS demyelination.We further examined that association between use of DMT and markers of viral infection.

6.4 Methods

6.4.1 Study design

As described elsewhere[19], the Ausimmune Study was a multicentre case-control study which recruited individuals at their first clinical diagnosis of CNS demyelination (FCD). Participating cases were aged 18 to 59 years old and resident in one of four study regions on the east coast of Australia. A subsequent longitudinal prospective cohort study, the Ausimmune Longitudinal (AusLong) Study has continued to follow the case participants of the Ausimmune Study with high retention (84.6%) to five years following their initial participation in the Ausimmune Study. More patients received disease modifying therapy from baseline interview (58/279) to 5-year review (127/236). At study entry, 21% (58/279) patients accepted DMTs and most of them (51/58) used interferon-beta, while in the 5-year review, the proportion of patients accepted DMTs increased to 54% (127/236) and only 60% (74/127) used interferon-beta, with 23 (18%) and 12 (9%) used copaxone and natalizumab, respectively.

Of the 282 cases considered to be eligible as having a FCD, three cases were subsequently diagnosed with a non-MS disorder (one neuromyelitis optica, one Susac's Syndrome, and one a pineal germinoma). Of the remaining 279 cases, review of notes at 5 years led to 260 cases now considered to have a relapse-onset and 19 had progressive-onset disease. Amongst the relapse-onset cases, 219 had their FDE within the study recruitment period. Here we report on the data collected during the first 5 years of follow up.

6.4.2 Data collection

Participants completed questionnaires prior to, and during, a face to face interview with a study nurse on entry to the Ausimmune Study. History of IM ('have you ever had glandular fever?') at baseline. Use (yes/no) of disease-modifying therapy (DMT) were reported at annual review. Since only baseline DMTs were associated with viral biomarkers, we used baseline DMTs as the covariate in the primary multivariable model. However, longitudinal DMTs had a closer relationship with the clinical course, so a sensitivity analysis with longitudinal DMTs were performed. We found that associations between viral biomarkers and clinical course did not change materially when longitudinal DMTs were used (data not shown).Blood was taken by venepuncture; aliquots of serum and whole blood were stored at -80 ℃ until completion of the Ausimmune Study.

Measurement of EBV/HHV6 variables

As previously described[5], serological EBV and HHV6 parameters were measured in blood taken at the initial interview for the Ausimmune Study in a subset of 205 cases, with 167 having their FDE during recruitment period. A comparison of the data between those patients who did and those who did not have the data of viral biomarkers showed that the two groups were very similar (supplementaltable 1). As a result, our sample is likely representative of the entire AusLong population. Quantitative IgG antibody titres to EBV viral capsid antigen (VCA) were measured by automated enzyme immunoassay (Star Corp, Stillwater, MN) and antibodies to EBNA complex and early antigen (diffuse and restricted, EA-D and EA-R, respectively) by immunofluorescence assay. EBV DNA load was quantified in whole blood using the BWRF-1 primers as described elsewhere.[20] Serum anti-HHV6 IgG titres were measured using an analogous assay (Panbio).[17] Anti-HHV6 IgG was used as a marker of HHV6 exposure, while HHV6 DNA and anti-HHV6 IgM were used as markers of HHV6 reactivation or new infection.All viral assays were performed critically as the illustration from manufacturer, but some biomarkers might still be inaccurate and it was difficult for us to control this potential measurement error. However, there was no evidence that the potential measurement error was

significantly differential by disease activity. So we believed that the potential measurement error of viral biomarkers would not influence the validity of the result.

Measurement of conversion to MS and relapse

Conversion to MS was defined primarily as the occurrence of a second clinical demyelinating event, thus satisfying the diagnostic requirements of dissemination in time and space, or a single clinical episode plus para-clinical evidence, as per the 2005 McDonald criteria (a minority of cases were diagnosed as 2010 McDonald criteria, following neurologist-ordered MRI assessment (n=20)).[21 22] Conversion to MS was reported at annual review or assessed by neurologist at face-to-face review, as well as derived from review of neurological records. A relapse was defined according to the 2001 McDonald Criteria as the acute or subacute appearance or reappearance of a neurological abnormality (lasting at least 24 hours) in the absence of other potential explanatory factors.[22] Relapses were reported at annual review and confirmed by review of the clinical notes, and only relapses which were diagnosed and verified by the study neurologist were included in this analysis.

Measurement of disability progression

Disability was assessed using the EDSS at 5-year review. For relapsing-onset cases, annualised change of EDSS was calculated with EDSS at 5-year review divided by the duration between date of 5-year review and date of FDE with the assumption that EDSS was 0 before FDE. However, for PPMS cases, accurate measurement of date of FDE was extremely difficult, so annualised change of EDSS was calculated with EDSS at 5-year review minus EDSS at study entry and divided by the duration between date of 5-year review and date of study entry.

It is acknowledged that the increase of EDSS is not linear, however, the AusLong study participants were people with a first clinical diagnosis of demyelination at study entry and 87.6% had an EDSS score ≤ 4 at the 5-year review. Previous research has suggested that the disability progression is comparative linear when EDSS was ≤ 4. Time to particular EDSS hallmarks were less suitable as the participants were not

assessed frequently enough by neurologists to reliably determine the date participants reached an EDSS of three or four. Therefore, annualised change of EDSS in the AusLong study was the best measure of disability progression.

6.4.3 Data analysis

Mean and standard deviation, median and interquartile range were used as descriptive statistics. Data management: participants stating that they did not know if they had had IM were coded as "no history of IM". Body mass index (BMI) was calculated as weight (kg) divided by squared height (m^2).

Ordinal logistic regression was used to assess the associations between baseline disease modifying therapy (DMT) and the levels of viral biomarkers. To verify the results of the association between baseline DMT and immune response to anti-EBNA IgG, we also analysed data from MS cases from the Tasmanian MS case-control study(n=136) and Southern Tasmanian Multiple Sclerosis Longitudinal (MSL) Study (n=198).[10 23] In the Tasmanian MS case-control study, anti-EBNA IgG titres were measured using an ELISA assay (Panbio, Brisbane, Australia). In the MSL study, anti-EBNA IgG titres were measured using an indirect immunofluorescence assay (IFA) (Panbio Inc., Columbia, MD, USA).

The effect of predictors on time-to-conversion and time-to-relapse was estimated using Cox proportional hazards models. Since Cox proportional hazards models allow cases to be censored before reaching endpoint, all available cases (n=279) but not those retained at 5-year review(n=236) were included in analyses. All analyses were adjusted for age, sex, study site, and for time to relapse analysis, baseline use of DMT was further adjusted. Since baseline 25(OH)D levels and history of tobacco smoking were not associated with MS clinical course (data not shown), they were not considered as covariates here. All covariates of interest satisfied the proportional hazards assumption. For conversion to MS analysis, the starting point was the date of first recorded symptom (FDE) onset. For better accuracy in determining the date of FDE, only relapsing-onset cases who had their FDE during the recruitment period (n=219) were selected for the analysis. Since we did not obtain the viral parameters

and IM information for all cases, only 167 and 216 cases were available for viral parameter and IM analysis, respectively.

For the relapse analysis, multiple relapses that occurred for the same case were regarded as independent observations on the assumption that time until a prior event does not affect the composition of the risk set for a subsequent event. Although cases with more severe disease were potentially going to have a higher relapse rate but dissemination in space and time criteria for relapse means that a relapse shouldn't affect the risk of a subsequent one. And all relapses included were all 30+ days apart, they should be fully independent. We also clustered the relapses when they happened in the same case to control the potential correlation. The relapse analysis included all available relapse onset cases (n=260), because the starting point of this analysis was the date of the event that brought the participants into the study. Not all cases provided information for viral parameters and IM, so only 194 and 256 of 279 cases were available for viral parameter and IM analysis, respectively.

Predictors of annualised change in EDSS were evaluated using linear regression adjusted for whether participants were having a relapse at the time of their 5-year review EDSS assessment. Because the annualised change in disability was highly skewed and for some cases the change of EDSS was zero, a log-transformation of the original score plus one was applied to satisfy linear regression assumptions of minimal heteroskedasticity. All means and coefficients, however, were back-transformed and presented on the original scale of the change in EDSS variable. As the EDSS is designed for those with a diagnosis of MS, we included only those who had been diagnosed as RRMS or PPMS by the 5-year review (n=186). Amongst the remaining 186 cases, 144 and 183 were available for viral parameters and IM analysis, respectively.

6.5 Results

6.5.1 Participant characteristics

The mean age of the 279 participants with FCD in the Ausimmune Study was 38.7 years and around roughly three-quarters were female (Table 1). Among the total cohort, 211 converted to MS and experienced 458 relapses. The distribution of categories of antibody titres at baseline for case participants are shown in Table 1.EBV and HHV-6 DNA were detected in only a small proportion of all cases (~10%). Other characteristics of the total cohort and cases with an FDE during the recruitment period are shown in Table 1.

Table 6.1 Demographic and clinical characteristics of the total cohort, those with an FDE during the recruitment period and those who retained at 5-year review and converted to MS and of the Ausimmune Study

	Total cohort n(%)	Cases with an FDE during the recruitment periodn(%)
Total	279	219
Female	214 (76.70)	168 (76.71)
Study site		
QLD	91 (32.62)	59 (26.94)
NSW	39 (13.98)	66 (30.14)
VIC	69 (24.73)	52 (23.74)
TAS	80 (28.67)	42 (19.18)
MS course at study entry		
FDE relapse-onset	260(93.19)	
Progressive onset	19(6.81)	
DMT used at baseline	58 (20.79)	44 (20.09)
Conversion to MS during study	211 (75.63)	159 (72.60)
Number of relapses during study	458	347
anti-EBNA IgG titre [a]		
≤40	44 (21.46)	37 (22.16)
160	89 (43.41)	76 (45.51)
≥640	72 (35.12)	54 (32.34)
anti-EBV-VCA IgG titre [a]		
≤160	39 (19.02)	33 (19.76)
640	110 (53.66)	87 (52.10)
≥1280	56 (27.32)	47 (28.14)
anti-EBV-EA-R IgG titre [a]		
≤10	69 (33.66)	60 (35.93)
40	66 (32.20)	52 (31.14)
≥160	70 (34.15)	55 (32.93)
anti-EBV-EA-D IgG titre [a]		

0	45 (21.95)	36 (21.56)
10	54 (26.34)	43 (25.75)
40	81 (39.51)	68 (40.72)
160-640	25 (12.20)	20 (11.98)
Serum EBV DNA detection		
Negative	180 (87.80)	145 (86.83)
Positive	25 (12.20)	22 (13.17)
anti-HHV6 IgG titre [a]		
≤40	65 (31.86)	51 (30.72)
160	95 (46.57)	77 (46.39)
≥640	44 (21.57)	38 (22.89)
anti-HHV6 IgM titre [a]		
0	193 (95.07)	156 (94.55)
20	7 (3.45)	6 (3.64)
40	3 (1.48)	3 (1.82)
Serum HHV6 DNA detection		
Negative	177 (86.34)	142 (85.03)
Positive	28 (13.66)	25 (14.97)
		Mean (SD; Range)
Age at study entry, years	38.72(9.75;18,58)	38.26 (9.68; 18, 58)
MS duration from first symptoms to 5-year review, years [b]	6.13 (2.49; 0.27, 21.2)	5.48 (1.32; 0.27, 8.08)
		Median (IQR)
EDSS at 5-year study review	2 (1; 2.5)	1.5 (1; 2.5)
Annualised change of EDSS	0.27 (0.17; 0.43)	0.27 (0.18; 0.41)

a: 205 cases had values for serum biomarkers; b: 236 cases retained in the 5-year review

6.5.2 Association between baseline DMT and anti-EBV/HHV6

There was no association between anti-EBNA IgG and anti-HHV6 IgG (r=0.11, p=0.26) in all cases. EBV or HHV6 biomarkers were not associated with a history of IM (p>0.05).

With the ordinal logistical regression, those who used DMT before study entry were less likely to have higher levels of anti-EBNA IgG (OR 0.44, 95% CI: 0.23 - 0.84; p=0.01), the association was robust for adjustment for age and sex (OR 0.39, 95% CI: 0.19 - 0.82; p=0.014) and enhanced after adjustment for age, sex, study centre and whether diagnosed as MS or not (OR 0.35, 95% CI: 0.16 - 0.76; p=0.008). However, there was no association in the cases of the Tasmanian case-control study (p=0.76) or MSL study (p=0.73), after adjustment for age and sex. Neither were other anti-EBV or anti-HHV6 biomarkers associated with baseline use of DMT.

6.5.3 Association between viral load & serological parameters of EBV and HHV6 and the hazard of conversion to MS

We found no associations between EBV-VL, serological antibodies (anti-EA-R IgG, anti-EA-D IgG, anti-EBNA1 IgG, anti-VCA IgG), or a history of IM with the risk of conversion to MS (Table 2). We found a significant association between HHV6-VL and conversion to MS in the univariable analysis; however, after adjustment (age, sex, study site, BMI and baseline DMT), the association was no longer statistically significant (p=0.36). There was no association between anti-HHV6 IgM or anti-HHV6 IgG and risk of conversion to MS (Table 2).

Table 6.2Associations of baseline levels of viral markers and the hazard of conversion to MS in cases with an FDE during the recruitment period

		Univariable analysis		Multivariable analysis	
	Conversions/person-years (rate)	HR (95% CI)	p	aHR (95% CI)	p
EBV DNA					
Negative	109/316.85 (0.34)	1.00 (Reference)		1.00 (Reference)	
Positive	16/51.57 (0.31)	0.90 (0.55, 1.49)	0.69	1.11 (0.70, 1.74)	0.66
anti-EA-R IgG					
≤10	47/131.18 (0.36)	1.00 (Reference)		1.00 (Reference)	
40	37/117.04 (0.32)	0.91 (0.59, 1.41)	0.66	0.88 (0.57, 1.35)	0.54
≥160	41/120.2 (0.34)	0.96 (0.64, 1.44)	0.85	0.91 (0.59, 1.42)	0.68
Trend			0.84		0.67
anti-EA-D IgG					
0	26/73.87 (0.35)	1.00 (Reference)		1.00 (Reference)	
10	34/89.54 (0.38)	1.00 (0.58, 1.70)	0.99	0.83 (0.47, 1.47)	0.53
40	50/168.08 (0.3)	0.86 (0.51, 1.44)	0.56	0.80 (0.46, 1.40)	0.43
160-640	15/36.93 (0.41)	1.10 (0.55, 2.18)	0.79	1.02 (0.53, 2.00)	0.94
Trend			0.79		0.64
anti-EBNA IgG					
≤40	31/59.44 (0.52)	1.00 (Reference)		1.00 (Reference)	
160	54/182.89 (0.3)	0.61 (0.38, 0.98)	***0.043***	0.69 (0.42, 1.13)	0.14
≥640	40/126.09 (0.32)	0.63 (0.39, 1.04)	0.07	0.76 (0.48, 1.20)	0.23
Trend			0.10		0.32
anti-VCA IgG					
≤160	28/49.75 (0.56)	1.00 (Reference)		1.00 (Reference)	
640	62/211.24 (0.29)	0.61 (0.39, 0.97)	***0.038***	0.66 (0.41, 1.05)	0.08
≥1280	35/107.43 (0.33)	0.63 (0.39, 1.03)	0.06	0.48 (0.29, 0.81)	***0.005***
Trend			0.10		0.006
IM (those who did not know whether they had a history of IM were coded as no history of IM)					
No	114/348.19 (0.33)	1.00 (Reference)		1.00 (Reference)	
Yes	42/133.02 (0.32)	0.97 (0.68, 1.37)	0.86	0.89 (0.61, 1.3)	0.54
IM (excluding those who did not know whether they had a history of IM)					
No	102/318.73 (0.32)	1.00 (Reference)		1.00 (Reference)	

Yes	42/133.02 (0.32)	0.99 (0.7, 1.42)	0.98	0.92 (0.63, 1.34)	0.66
HHV6 DNA					
Negative	100/329.89 (0.3)	1.00 (Reference)		1.00 (Reference)	
Positive	23/38.02 (0.6)	1.79 (1.14, 2.83)	***0.012***	1.29 (0.74, 2.28)	0.36
anti-HHV6 IgM					
0	116/333.16 (0.35)	1.00 (Reference)		1.00 (Reference)	
20	3/27.44 (0.11)	0.39 (0.15, 0.97)	***0.044***	0.33 (0.15, 0.71)	***0.005***
40	2/5.83 (0.34)	0.81 (0.28, 2.37)	0.70	0.38 (0.13, 1.16)	0.09
Trend			0.17		***0.010***
anti-HHV6 IgG					
≤40	33/141.57 (0.23)	1.00 (Reference)		1.00 (Reference)	
160	60/134.36 (0.45)	1.62 (1.03, 2.54)	***0.035***	1.44 (0.89, 2.35)	0.14
≥640	29/91.5 (0.32)	1.20 (0.75, 1.91)	0.46	1.13 (0.70, 1.82)	0.63
Trend			1.00		0.90

Adjusted for age, sex, study site, BMI and baseline DMT in the primary analysis. Figures in boldface denote statistical significance ($p<0.05$).
Abbreviations: HR, hazard ratio; aHR, adjusted hazard ratio; IM, infectious mononucleosis.

6.5.4 Association between viral load & serological parameters of EBV and HHV6 and the hazard of relapse

There was no association between EBV-VL and relapse risk (Table 3). However, we identified a dose-dependent association between anti-EA-R IgG and hazard of relapse. There were no associations between other serological EBV variables and risk of relapse. However, a history of IM was associated with a 50% increase in the hazard of relapse.

There were no associations between detection of HHV6 DNA or serological parameters (anti-HHV6 IgG, anti-HHV6 IgM) and the risk of relapse (Table 3).

Table 6.3Associations of baseline levels of viral markers and the hazard of relapse in all relapsing onset cases

		Univariable analysis		Multivariable analysis	
	Relapses/person-years (rate)	HR (95% CI)	p	aHR (95% CI)	p
EBV DNA					
Negative	298/930.45 (0.32)	1.00 (Reference)		1.00 (Reference)	
Positive	48/139.27 (0.34)	1.09 (0.74, 1.62)	0.66	1.16 (0.84, 1.6)	0.35
anti-EAR IgG					
≤10	98/374.07 (0.26)	1.00 (Reference)		1.00 (Reference)	
40	97/342.77 (0.28)	1.07 (0.80, 1.45)	0.64	1.04 (0.78, 1.39)	0.80
160-640	151/352.89 (0.43)	1.49 (1.03, 2.17)	***0.034***	1.43 (1.05, 1.94)	***0.022***
Trend			***0.039***		***0.023***
anti-EA-D IgG					
0	73/232.11 (0.31)	1.00 (Reference)		1.00 (Reference)	
10	84/277.96 (0.30)	0.90 (0.62, 1.29)	0.55	0.92 (0.64, 1.32)	0.64
40	142/438.73 (0.32)	0.99 (0.66, 1.50)	0.98	1.08 (0.74, 1.58)	0.69
160-640	47/120.93 (0.39)	1.17 (0.61, 2.28)	0.64	1.22 (0.70, 2.10)	0.48

Trend			0.50		0.33
anti-EBNA IgG					
≤40	77/228.79 (0.34)	1.00 (Reference)		1.00 (Reference)	
160	156/468.11 (0.33)	0.94 (0.66, 1.34)	0.73	1.12 (0.80, 1.56)	0.51
≥640	113/372.82 (0.30)	0.90 (0.59, 1.38)	0.63	1.05 (0.72, 1.55)	0.79
Trend			0.65		0.86
anti-VCA IgG					
≤160	70/196.02 (0.36)	1.00 (Reference)		1.00 (Reference)	
640	160/582.65 (0.27)	0.82 (0.54, 1.23)	0.33	0.81 (0.59, 1.11)	0.20
≥1280	116/291.05 (0.40)	1.06 (0.73, 1.53)	0.78	0.95 (0.67, 1.33)	0.75
Trend			0.61		0.85
IM (those who did not know whether they had a history of IM were coded as no history of IM)					
No	284/1037.02 (0.27)	1.00 (Reference)		1.00 (Reference)	
Yes	168/373.69 (0.45)	1.48 (1.10, 1.99)	***0.010***	1.52 (1.16, 2.00)	***0.003***
IM (excluding those who did not know whether they had a history of IM)					
No	263/934.48 (0.28)	1.00 (Reference)		1.00 (Reference)	
Yes	168/373.69 (0.45)	1.46 (1.08, 1.98)	***0.014***	1.51 (1.15, 2)	***0.003***
HHV6 DNA					
Negative	280/909.59 (0.31)	1.00 (Reference)		1.00 (Reference)	
Positive	66/160.14 (0.41)	1.26 (0.82, 1.93)	0.29	1.12 (0.73, 1.71)	0.61
anti-HHV6 IgM					
0	332/1003.29 (0.33)	1.00 (Reference)		1.00 (Reference)	
20	4/37.86 (0.11)	0.39 (0.22, 0.69)	***0.001***	0.48 (0.30, 0.78)	***0.003***
40	4/13.45 (0.30)	0.79 (0.47, 1.32)	0.37	0.56 (0.32, 1.01)	0.054
Trend			0.09		***0.011***
anti-HHV6 IgG					
≤40	112/337.88 (0.33)	1.00 (Reference)		1.00 (Reference)	
160	163/498.52 (0.33)	0.97 (0.64, 1.47)	0.88	0.95 (0.65, 1.37)	0.78
≥640	66/223.70 (0.30)	0.87 (0.57, 1.33)	0.52	0.92 (0.61, 1.39)	0.70
Trend			0.47		0.73

Adjusted for age, sex, BMI study centres and disease modifying therapyat baseline.
Figures in boldface denote statistical significance ($p<0.05$).
Abbreviations: HR, hazard ratio; aHR, adjusted hazard ratio; IM, infectious mononucleosis.

6.5.5 Association between immune response to EBV/HHV6 and clinical disability progression

We examined the association between baseline markers of viral infection and annualised change of EDSS from the date of symptom onset to the 5-year review for participants who had been diagnosed with RRMS or PPMS by 5th year review (n=144). There was a statistically significant increase in annualised EDSS progression as anti-HHV6 IgG titre increased (EDSS change per year of 0.37 for those with the highest anti-body level (≥640) compared to a 0.26 change per year for those with an anti-body level of 40) (Table 4).

Table 6.4Associations of baseline levels of viral markers and the clinical disability progression among those who converted to MS at 5-year review

		Model [a]		Model [b]	
	No. (%)	β (95% CI)	P	β (95% CI)	P
EBV DNA					
Negative	127 (88.19)	0.29 (0.25, 0.33) [c]		0.29 (0.26, 0.33) [c]	
Positive	17 (11.81)	+0.02 (-0.09, 0.14)	0.73	-0.01 (-0.12, 0.11)	0.88
anti-EAR IgG					
≤10	50 (34.72)	0.29 (0.23, 0.35) [c]		0.30 (0.24, 0.36) [c]	
40	45 (31.25)	+0.02 (-0.08, 0.11)	0.73	+0.02 (-0.07, 0.11)	0.68
160-640	49 (34.03)	0.00 (-0.09, 0.09)	0.92	-0.04 (-0.13, 0.05)	0.41
Trend			0.92		0.44
anti-EA-D IgG					
0	32 (22.22)	0.32 (0.24, 0.39) [c]		0.32 (0.24, 0.40) [c]	
10	40 (27.78)	+0.03 (-0.08, 0.13)	0.61	+0.03 (-0.08, 0.14)	0.58
40	56 (38.89)	-0.06 (-0.16, 0.04)	0.25	-0.06 (-0.17, 0.04)	0.21
160-640	16 (11.11)	-0.06 (-0.20, 0.07)	0.37	-0.08 (-0.22, 0.07)	0.30
Trend			0.21		0.17
anti-EBNA IgG					
≤40	32 (22.22)	0.29 (0.22, 0.37) [c]		0.29 (0.21, 0.37) [c]	
160	60 (41.67)	+0.01 (-0.09, 0.11)	0.85	0.02 (-0.08, 0.12)	0.64
≥640	52 (36.11)	-0.01 (-0.11, 0.09)	0.83	-0.01 (-0.11, 0.09)	0.90
Trend			0.79		0.80
anti-VCA IgG					
≤160	27 (18.75)	0.25 (0.17, 0.34) [c]		0.26 (0.18, 0.34) [c]	
640	78 (54.17)	+0.06 (-0.04, 0.16)	0.23	+0.07 (-0.03, 0.17)	0.16
1280-5120	39 (27.08)	+0.03 (-0.08, 0.14)	0.60	-0.02 (-0.13, 0.09)	0.78
Trend			0.70		0.74
IM (those who did not know whether they had a history of IM were coded as no history of IM)					
No	135 (73.77)	0.30 (0.26, 0.34) [c]		0.30 (0.26, 0.34) [c]	
Yes	48 (26.23)	+0.02 (-0.05, 0.10)	0.55	+0.02 (-0.06, 0.10)	0.60
IM (excluding those who did not know whether they had a history of IM)					
No	120 (71.43)	0.31 (0.27, 0.35) [c]		0.31 (0.27, 0.35) [c]	
Yes	48 (28.57)	+0.01 (-0.06, 0.09)	0.73	+0.01 (-0.07, 0.09)	0.83
HHV6 DNA					
Negative	120 (83.33)	0.30 (0.26, 0.34) [c]		0.30 (0.26, 0.34) [c]	
Positive	24 (16.67)	-0.04 (-0.13, 0.06)	0.47	-0.04 (-0.14, 0.06)	0.46
anti-HHV6 IgM					
0	137 (96.48)	0.29 (0.25, 0.33) [c]		0.29 (0.25, 0.33) [c]	
20	3 (2.11)	-0.10 (-0.33, 0.14)	0.42	-0.03 (-0.28, 0.23)	0.84
40	2 (1.41)	+0.43 (0.02, 0.85)	0.039	+0.39 (-0.01, 0.80)	0.057
Trend			0.11		0.08
anti-HHV6 IgG					
≤40	43 (30.07)	0.28 (0.22, 0.35) [c]		0.26 (0.19, 0.32) [c]	
160	69 (48.25)	-0.00 (-0.09, 0.08)	0.95	+0.03 (-0.06, 0.11)	0.55
≥640	31 (21.68)	+0.06 (-0.05, 0.16)	0.29	+0.11 (0.00, 0.22)	0.051
Trend			0.22		***0.044***

Results are presented as the mean (95% CI) of reference level of the predictor, then coefficient (95% CI) of other levels relative to reference. Significant results were denoted in bold and italic font.
a: adjusted for relapse or not in the 5-year review; b: further adjusted for sex, age, study site, baseline BMI and baseline disease modifying therapy; c: annualised change of EDSS in reference group.

6.6 Discussion

Using one of the most comprehensive prospective studies of MS cases in the early stage of disease, we evaluated the roles of EBV and HHV6 infection in MS clinical course, including conversion to MS, relapse and disability progression. We found that a history of IM and higher levels of anti-EA-R IgG (≥ 640) at baseline were associated with a 50% greater hazard of relapse but this was not significantly associated with risk of conversion to MS. There were no other robust associations between viral load or serological markers of EBV infection and MS clinical course. Higher anti-HHV6 IgG titre was associated with a greater annualised change in disability, but was not associated with risk of conversion or hazard of relapse. Anti-HHV6 IgM and HHV6 DNA were not associated with MS clinical course.

The features of latency and reactivation of EBV led to the hypothesis that reactivation may be one factor in initiating relapses of MS. Higher levels of anti-EA-R IgG at baseline were associated with increased hazard of relapses, but no association of conversion to MS was seen. The disparate findings were unexpected, as there is some overlap between the two analyses. However, the relapse analysis included all people with RRMS, included all relapses and used the date of FCD as the starting point. The conversion to MS analysis excluded those where their FDE occurred prior to the recruitment period (n=41) because there was concern about measurement error for these people, and used FDE as the starting point. While conversion to MS was often determined by the second relapse, it could also occur by MRI lesions (n=20). One previous cohort study followed 19 patients for 1 year also found that patients with exacerbations during the follow-up period had an increasing of anti-EA-R IgG levels.[24] However, most studies showed no associations between *active* EBV infection and clinical course of MS, and our study also found no associations of anti-EA-D IgG and EBV DNA at baseline and MS clinical course. Our previous MSL study found

that reactivation of EBV and HHV6 infection was not associated with relapse, and evidence for active EBV infection was seen in only a minority of the study cohort at any time point (detection of EBV DNA 29%; HHV6 DNA 1.8%).[25] Some other groups' research have also shown no associations.[26 27] Moreover, the absence of association of active EBV infection and MS onset suggested that no effects of active EBV infection with MS clinical course.[5 7 28 29]

In agreement with our work, one recent study[9] (BENEFIT) with 468 CIS cases followed for five years found that the risk of conversion to MS was similar in cases of highest and lowest category of baseline anti-EBNA1 IgG (HR 1.14, 95% CI: 0.76-1.72). Three other studies that focused on disease activity also found no associations with anti-EBNA1 IgG levels at baseline.[10 11 16]No association between EBV infection and annualised change of EDSS was observed in our study, which is similar from results of the BENEFIT study (β=0.02, p=0.80).[9] One other study found that anti-EBNA-1 IgG levels were positively associated with change in EDSS (r=0.3, p=0.004) measured over 5 years, but they did not examine and account for potential confounding variables.[12]

A history of IM was associated with a higher hazard of relapse in the present study. While IM is viewed as a risk factor for onset of MS[4], to date, no study has focused on the association between a history of IM and MS clinical course. However, the absence of internal consistency between related outcomes (conversion to MS and change in EDSS) did give some concern as to the validity of the association with relapse.

In this study, anti-HHV6 IgG was associated with annualised change in disability, but not with conversion or relapse, while markers of active HHV6 infection were not associated with MS clinical course. This does not align with our previous MSL study[10] which found that a higher anti-HHV6 IgG was associated with a higher risk of relapse but not annualised change in disability in a sample of established MS followed for 2.5 years. It is hard to understand the reasons for the differential findings, although timing of assessment early in the MS course as opposed to established MS and low rates of DMT use are possible explanations. One other explanation is that the MSL

study took blood 6 monthly so would be more likely to detect a short term elevation in viral markers just before relapse than this study. A few studies showed some associations between HHV6 parameters and MS clinical course. One prospective study that followed 301 MS patients for two years found that anti-HHV6 IgG levels elevated to the highest level around two weeks prior to relapse ($p=0.001$)[17].Some significant limitations in this study influenced the interpretation of the results: patients were all treated with interferon-beta, natalizumab or GA, not controlling for potential confounders such as tobacco smoking, vitamin D levels, genetic background, and no statement of the inclusion criteria. Other research showed a significant association between HHV6 parameters and disease activity on MRI.[18 30]Only a few studies have evaluated the role of *active* HHV6 infection in the clinical course of MS, and the findings have been inconsistent. Our MSL study showed that anti-HHV6 IgM and HHV6 viral load were not associated with the hazard of relapses or disability progression.[25]Berti et al found a higher number of cases to be HHV6 DNA positive during relapses (4/18 vs. 11/197, $p=0.008$), using 215 serum samples from 59 MS patients.[30] However, this study failed to control for potential confounders, and the short duration of follow-up (5 months) may limit its generalisability.

We observed that those who used DMTs at baseline were less likely to have higher levels of anti-EBNA IgG, which was an interesting finding suggesting that immune response to EBV could be down-regulated through DMTs. However, we could not replicate this significant association in the Tasmanian case-control and MSL studies. One possible explanation is that these participants in the baseline Ausimmune Study were incident cases with FCD, while participants in the other two studies were prevalent cases that were diagnosed as MS and in many cases had been on DMTs for many years. Two other studies also found that anti-EBNA levels did not fluctuate materially when DMTs were used, however, sample size in these studies were small (both studies: n=20) and all were well established MS cases (disease duration 10 years).[31 32]

Strengths of our study include the use of a prospective longitudinal study design following cases with a first diagnosis of CNS demyelination. We had a high retention

rate at 5 year follow-up (84.6%), and a high rate (76%) of conversion to MS among those follow-up. A limitation was that levels of anti-EBV/HHV6 and viral load were only measured at baseline, so investigation of the association between longitudinal immune response to HHV6/EBV and MS clinical course was impossible. As previous studies have suggested, anti-EBV/HHV6 IgG levels were comparatively stable over time,[33 34] our capacity to detect associations was limited when active viral measures and disease activity were examined. So we cannot exclude the possibility that some results were produced by chance, replication in other similar cohorts is therefore desirable. Another limitation was that IM was happened years prior to study entry, and it was difficult for us to detect whether information about IM was accurately recorded at baseline interview. Although IM was decoded in two ways (those who did not know whether they had a IM before was decoded as no history of IM and missing), we still could not control the influence of recall bias fully. Dates of FDE and some relapses prior to study entry may be inaccurate especially for progressive-onset cases, so for conversion to MS analysis, we have restricted to cases with date of FDE during study entry in the, and for relapse analysis, date of FCD was set as the starting time. Using annualised change of EDSS to measure the disability progression was also a limitation in the study, due to the non-linear distribution of EDSS. But in AusLong study, annualised change of EDSS is the most suitable variable to measure disability progression since most patients were still benign at 5-year review with EDSS ≤ 4, and some research in MSBase has suggested that EDSS was comparatively linear in the lower range.

Since patients in AusLong study were recruited from relatively contemporary populations with a standardised protocol, birth cohort effect had a relatively less influence on the results. However, we still performed a sensitivity analysis based on the date of birth of the cases (1947-1969, 1961-1970, 1971-1988), and no significant interaction between exposures of interests and date of birth were found (data not shown).

Our data provides some evidence that elevated levels of anti-EAR IgG and a history of IM are associated with the subsequent hazard of relapses, and anti-HHV6 with

disability progression. Overall, epidemiological studies do not support associations with markers of *active* EBV/HHV6 infection and clinical course, while the evidence on markers of latent infection is merely inconclusive.

6.7 Postscript

This chapter showed some associations between EBV/HHV6 infections and MS disease activity. The next chapter will examine whether stressful life events could influence MS clinical course.

6.8 Reference

1. Ramagopalan SV, Dobson R, Meier UC, et al. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet neurology* 2010;9(7):727-39. doi: 10.1016/S1474-4422(10)70094-6
2. Mechelli R, Annibali V, Ristori G, et al. Multiple sclerosis etiology: beyond genes and environment. *Expert review of clinical immunology* 2010;6(3):481-90. doi: 10.1586/eci.10.11
3. Almohmeed YH, Avenell A, Aucott L, et al. Systematic review and meta-analysis of the sero-epidemiological association between Epstein Barr virus and multiple sclerosis. *PloS one* 2013;8(4):e61110. doi: 10.1371/journal.pone.0061110 [published Online First: 2013/04/16]
4. Handel AE, Williamson AJ, Disanto G, et al. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PloS one* 2010;5(9) doi: 10.1371/journal.pone.0012496
5. Lucas RM, Ponsonby AL, Dear K, et al. Current and past Epstein-Barr virus infection in risk of initial CNS demyelination. *Neurology* 2011;77(4):371-9. doi: 10.1212/WNL.0b013e318227062a [published Online First: 2011/07/15]
6. Virtanen JO, Farkkila M, Multanen J, et al. Evidence for human herpesvirus 6 variant A antibodies in multiple sclerosis: diagnostic and therapeutic implications. *Journal of neurovirology* 2007;13(4):347-52. doi: 10.1080/13550280701381332
7. Kuusisto H, Hyoty H, Kares S, et al. Human herpes virus 6 and multiple sclerosis: a Finnish twin study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(1):54-8. doi: 10.1177/1352458507080063
8. Xu Y, Linde A, Fredrikson S, et al. HHV-6 A- or B-specific P41 antigens do not reveal virus variant-specific IgG or IgM responses in human serum. *Journal of medical virology* 2002;66(3):394-9.
9. Munger KL, Fitzgerald KC, Freedman MS, et al. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. *Neurology* 2015;85(19):1694-701. doi: 10.1212/wnl.0000000000002099 [published Online First: 2015/10/11]
10. Simpson S, Jr., Taylor B, Dwyer DE, et al. Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis. *Multiple sclerosis*

(Houndmills, Basingstoke, England) 2012;18(6):799-806. doi: 10.1177/1352458511428081 [published Online First: 2011/11/16]
11. Lunemann JD, Tintore M, Messmer B, et al. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Annals of neurology* 2010;67(2):159-69. doi: 10.1002/ana.21886
12. Farrell RA, Antony D, Wall GR, et al. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. *Neurology* 2009;73(1):32-8. doi: 10.1212/WNL.0b013e3181aa29fe [published Online First: 2009/05/22]
13. Lünemann JD, Tintoré M, Messmer B, et al. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Annals of neurology* 2010;67(2):159-69. doi: 10.1002/ana.21886
14. Kvistad S, Myhr KM, Holmoy T, et al. Antibodies to Epstein-Barr virus and MRI disease activity in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014 doi: 10.1177/1352458514533843
15. Zivadinov R, Zorzon M, Weinstock-Guttman B, et al. Epstein-Barr virus is associated with grey matter atrophy in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2009;80(6):620-5. doi: 10.1136/jnnp.2008.154906
16. Horakova D, Zivadinov R, Weinstock-Guttman B, et al. Environmental factors associated with disease progression after the first demyelinating event: results from the multi-center SET study. *PloS one* 2013;8(1):e53996. doi: 10.1371/journal.pone.0053996
17. Ortega-Madueno I, Garcia-Montojo M, Dominguez-Mozo MI, et al. Anti-human herpesvirus 6A/B IgG correlates with relapses and progression in multiple sclerosis. *PloS one* 2014;9(8):e104836. doi: 10.1371/journal.pone.0104836
18. Virtanen JO, Wohler J, Fenton K, et al. Oligoclonal bands in multiple sclerosis reactive against two herpesviruses and association with magnetic resonance imaging findings. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(1):27-34. doi: 10.1177/1352458513490545 [published Online First: 2013/06/01]
19. Lucas R, Ponsonby AL, McMichael A, et al. Observational analytic studies in multiple sclerosis: controlling bias through study design and conduct. The Australian Multicentre Study of Environment and Immune Function. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2007;13(7):827-39. doi: 10.1177/1352458507077174 [published Online First: 2007/09/21]
20. Lay ML, Lucas RM, Ratnamohan M, et al. Measurement of Epstein-Barr virus DNA load using a novel quantification standard containing two EBV DNA targets and SYBR Green I dye. *Virology journal* 2010;7:252. doi: 10.1186/1743-422x-7-252 [published Online First: 2010/09/24]
21. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Annals of neurology* 2005;58(6):840-6. doi: 10.1002/ana.20703 [published Online First: 2005/11/12]
22. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Annals of neurology* 2011;69(2):292-302. doi: 10.1002/ana.22366

23. van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *Bmj* 2003;327(7410):316. doi: 10.1136/bmj.327.7410.316
24. Wandinger K, Jabs W, Siekhaus A, et al. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. *Neurology* 2000;55(2):178-84.
25. Simpson S, Jr., Taylor B, Burrows J, et al. EBV & HHV6 reactivation is infrequent and not associated with MS clinical course. *Acta neurologica Scandinavica* 2014 doi: 10.1111/ane.12268 [published Online First: 2014/06/05]
26. Torkildsen O, Nyland H, Myrmel H, et al. Epstein-Barr virus reactivation and multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2008;15(1):106-8. doi: 10.1111/j.1468-1331.2007.02009.x
27. Buljevac D, van Doornum GJ, Flach HZ, et al. Epstein-Barr virus and disease activity in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2005;76(10):1377-81. doi: 10.1136/jnnp.2004.048504 [published Online First: 2005/09/20]
28. Tao C, Simpson Jr S, Taylor BV, et al. Association between human herpesvirus & human endogenous retrovirus and MS onset & progression. *Journal of the neurological sciences* 2017;372:239-49. doi: http://dx.doi.org/10.1016/j.jns.2016.11.060
29. Cocuzza CE, Piazza F, Musumeci R, et al. Quantitative detection of epstein-barr virus DNA in cerebrospinal fluid and blood samples of patients with relapsing-remitting multiple sclerosis. *PloS one* 2014;9(4):e94497. doi: 10.1371/journal.pone.0094497 [published Online First: 2014/04/12]
30. Berti R, Brennan MB, Soldan SS, et al. Increased detection of serum HHV-6 DNA sequences during multiple sclerosis (MS) exacerbations and correlation with parameters of MS disease progression. *Journal of neurovirology* 2002;8(3):250-6. doi: 10.1080/13550280290049615-1 [published Online First: 2002/06/08]
31. Castellazzi M, Delbue S, Elia F, et al. Epstein-Barr Virus Specific Antibody Response in Multiple Sclerosis Patients during 21 Months of Natalizumab Treatment. 2015;2015:901312. doi: 10.1155/2015/901312
32. Raffel J, Dobson R, Gafson A, et al. Multiple sclerosis therapy and Epstein-Barr virus antibody titres. *Multiple sclerosis and related disorders* 2014;3(3):372-4. doi: 10.1016/j.msard.2013.12.004 [published Online First: 2015/04/17]
33. Braun DK, Dominguez G, Pellett PE. Human herpesvirus 6. *Clinical microbiology reviews* 1997;10(3):521-67. [published Online First: 1997/07/01]
34. Henle W, Henle G, Andersson J, et al. Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 1987;84(2):570-4.

Table 6.6 Comparison of those patients who did and those who did not have the data of viral biomarkers

	Participants with biomarkers measured at baseline interview (n=205)	Participants without biomarkers measured at baseline interview (n=74)	P
Sex			
Male	46	19	0.57
Female	159	55	
CDMS			
No	42	20	0.25
Yes	163	54	
PPMS			
No	194	66	0.11
Yes	11	8	
Age at FCD	39.1 (9.8)	37.8 (9.7)	0.32

Chapter 7 Positive perceived stressful life events are associated with a lower hazard of relapses in early multiple sclerosis

7.1 preface

This chapter will address the fourth aim of the thesis – whether perceived positive or negative stressful life events could influence conversion to MS, time to relapses and disability progression with a cohort of patients with as first clinical diagnosis of CNS demyelination.

7.2 Abstract

Background: Despite significant evidence that psychological factors may influence disease course in many chronic disorders, few studies have investigated the association of stressful life events (SLEs) both negative and positive with the clinical course of multiple sclerosis (MS), especially the effect of positive SLEs.

Method: This prospective cohort study included data from 251 first episode of central nervous system demyelination subjects with SLE and clinical course variables measured prospectively up to 5–year post onset. SLEs were measured from 1 year prior to the event that brought subjects into the study to 5–year review. Conversion to MS and relapse were the primary study endpoints and were recorded by face-to-face neurological assessments and medical record reviews.

Results: A greater number (p=0.035), higher perceived intensity (p=0.010) and load (p=0.052) and a longer duration of the events (p=0.042) that were perceived as *positive* were consistently associated with a reduced hazard of relapses in a dose-dependent manner. Associations were less strong for conversion to MS. There were no associations between perceived *negative* SLEs and MS conversion or relapse risk. There were no associations between any SLEs variables and disability progression as measured by annualised change of expanded disability scale score.

Conclusion: We found consistent evidence that positive perceptions of SLEs were associated with a reduced risk of relapses, suggesting that positive SLEs and possibly an overall positive psychological well-being may reduce disease activity.

7.3 Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS). The wide disseminations of lesions within the CNS leads to the diversity of clinical symptoms, amongst the protean clinical features associated with MS; psychological dysfunctions are some of the most prevalent in MS patients[1] even in early disease stages[2]. Among the various risk factors implicated in MS, stress has been a frequently cited one. Current evidence[3-6] has consistently suggested that stressful life events (SLE) are associated with a higher risk of relapse or disability progression in people with MS. Certainly also they are potent modulators of comorbid depression and anxiety, and thus germane to patient quality of life.

A meta-analysis[3] including 14 studies found that previous stressful life events were associated with a higher risk of relapse (effect size (measured as (mean1−mean2)/σ) 0.53, 95% CI 0.40-0.65; $p<0.0001$). Although a subsequent systematic review[6] found the high level of heterogeneity in the measurement of stress and diagnosis of MS relapse precluding a statistically robust meta-analysis, the authors still concluded that there was a relatively consistent between SLEs and increased risk of MS onset or relapses. The association of SLEs and MS clinical course is of considerable interest given that psychological and well-being interventions aimed at improving coping may be potential non pharmacological interventions for MS. Indeed, a number of studies in MS patients have shown improvement of psychological and physical function after psychological interventions such as stress management therapy, and developing skills in active coping strategies[7-9].

Recent studies have mainly focused on whether negative SLEs predict MS relapses[10], with only a few studies exploring the effect of positive SLEs on MS clinical course[4 11 12]. One prospective cohort study[4], following 119 MS patients for 24 months, found that the absence of positive stressors was associated with a higher hazard of relapse (HR 3.14, CI 1.29-7.65). Some limitations in this study might influence our interpretation of the results: 1) Patients were comparatively benign, so the results may not be suitable to generalise to the whole MS populations, 2) At the last interview, around 50% of patients were lost, 3) diagnosis was not based on the MRI.Another study[11] following 121 MS patients for 48 weeks, found a reduced risk of new T2 lesions on MRI with increasing number of positive events (OR 0.74, 95% CI 0.55-0.99, p=0.04). This study was built on a RCT study (which was designed to examine the role of stress management therapy on MS relapses) with a post-hoc analysis, which may influence the interpretation of the results and generalisation to the general population of individuals with MS. Conversely; evidence on the association between SLEs and disability progression in MS patients remains limited. One prospective cohort study, following 36 MS patients for 100 weeks, found no association between SLEs and disability progression[12].

There is thus a gap in the literature for studies which examine the prospective association between both positively and negatively perceived stressful life events with the whole of MS clinical course, including conversion to active disease, relapses, and disability progression. In this prospective cohort study, we have evaluated the association between SLEs and clinical course in demyelinating disease patients at the

early stages of disease, in particular, whether there are differential effects between positive and negative SLEs.

7.4 Method

7.4.1 Study design

As described elsewhere[13], the Ausimmune study is a multicentre (Brisbane city, Newcastle city and surrounds, Geelong city and the Western Districts of Victoria, and the state of Tasmania) case-control study which recruited individuals diagnosed as a first clinical diagnosis of CNS demyelination (FCD). All participants at recruitment were aged 18 to 59 years. A subsequent prospective cohort study, the Ausimmune Longitudinal (AusLong) study followed these cases prospectively (retention rate 84.6% at 5 years). Participants completed an annual telephone review in addition to face-to-face reviews at 2-3 years and 5 years following their initial participation in the Ausimmune Study. Here we use the 5-year review data as the end point of our analysis as it was the last face to face review, i.e. the analysis period is from the first demyelinating event (FDE) to the 5-year review. Among the original 282 cases recruited to the Ausimmune study, 3 cases on review were found to not have an MS related event (one neuromyelitis optica, one Susac's Syndrome, and one pineal germinoma),and were removed leaving a total of 279 cases. Of these, 251 cases with recorded SLEs data during follow-up to the 5th year review constitute the cohort assessed in this study.

7.4.2 Measurement of MS clinical course

Conversion to MS was often but not always, based on the occurrence of a second clinical demyelinating episode, thus satisfying the diagnostic requirements of dissemination in time and space, or a single episode plus paraclinical evidence, as per the 2005 McDonald criteria[14] (a minority of cases were diagnosed following neurologist-ordered MRI (either at the 2/3 year or 5 year reviews) based on this latter criterion (n=20)). Conversion to MS was reported at annual review and cross-checked with neurological records.

A relapse was defined according to the 2001 McDonald Criteria[15] as the acute or subacute appearance or reappearance of a neurological abnormality (lasting at least 24 hours) in the absence of other potential explanatory factors. Relapses were reported at annual review or from neurological records, and only relapses which were diagnosed and verified by a neurologist were included in this analysis.

Disability was assessed by the Kurtzke Expanded Disability Status Scale (EDSS) at 5-year review. Annualised change in EDSS was calculated by taking the 5-year review EDSS and dividing by the duration between the day before FDE (date of FDE being reviewed by clinical notes and confirmed by consensus of the neurological group) and the 5-year review, with the assumption that EDSS was 0 at the day before FDE in those with relapse onset MS. However, for PPMS cases, accurate measurement of date of FDE was extremely difficult, so annualised change of EDSS was calculated with EDSS at 5-year review minus EDSS at study entry and divided by the duration between date of 5-year review and date of study entry.

It is acknowledged that the increase of EDSS is not linear, however, the AusLong study participants were people with a first clinical diagnosis of demyelination at study entry and 87.6% had an EDSS score ≤ 4 at the 5-year review. Previous research has suggested that the disability progression is comparative linear when EDSS was ≤ 4. Time to particular EDSS hallmarks were less suitable as the participants were not assessed frequently enough by neurologists to reliably determine the date participants reached an EDSS of three or four. Therefore, annualised change of EDSS in the AusLong study was the best measure of disability progression.

7.4.3 Measurement of stressful life events

SLEs were assessed at yearly intervals up to 5-year review using a modified version (excluding any illness leading to study participation) of the Social Readjustment Rating Scale developed by Holmes & Rahe (SRRS)[16]. The SLEs here included questions relating to the participant's employment or financial issues, gaining/losing family members, changing personal circumstances (habits, lifestyle, relationships, and employment), experiencing personal achievement/disappointment, health related issues, problems with the law or having a close friend/relative experience the same. Participants were asked to report the presence/absence of each type of event from 12 months prior to the event that brought cases to the AusImmune study to the 5th year review, the duration of each event (by providing the year and months that this event continued) and the perceived severity (a five point scale allowing the participants to assess the degree of perceived severity: -5 (worst) to +5 (best) Likert scale). The negative or positive valence of SLEs was determined by participants' ratings of the SLEs they experienced; SLEs rated >0 were classified as positive and SLEs<0were

classified as negative. Major negative SLEs[11] were defined as SLEs associated with physical damage or family loss to the patients. Major positive SLEs (mean perceived severity>3) were defined as SLEs associated with gaining a new family member, getting married or a personal achievement.

The total number of SLEs (SLE number) was calculated based on the total of reported SLE events. The total valence rating (SLE perceived severity) was calculated for both positive and negative events separately by addition of SLE Likert ratings. In addition, each SLE type was given a weight reflecting the amount of readjustment the event requires according to the load of each event (designed by Scully, devised by Holmes and Rahe and later reviewed by Scully et al. to reflect updates in culture change[17]), the total of these weights indicate a total load on coping capacity a given SLE might have on a participants of events (SLE load). A total duration of events (SLE duration) was calculated by summing the reported duration of each event. SLE number, SLE perceived severity, SLE load and SLE duration were calculated separately according to the perceived negative and positive events.

To establish whether the timing of the SLEs was important in relation to the outcomes, SLE measures were calculated for 12 months and 6 months prior to each review for conversion to MS/relapse analyses. This window was chosen because a shorter risk window would have resulted in insufficient SLE events while a longer risk window would have reduced the sample size because the questionnaire only recorded the SLE information as far back as 12 months prior to event that brought them into the study. For disability progression analyses, annualised SLE measures from the date of one year prior to FCD to 5-year review were used as predictor.

7.4.4 Measurement of other covariates

Our group previously demonstrated the significant association between body mass index (BMI) and MS clinical course, so BMI was also controlled in the multivariable model[18].Height and weight were measured by a study nurse at baseline review using a standardised protocol[13]. Body mass index (BMI) was calculated as weight (kg) divided by squared height (m^2). Other baseline measurements included the use of disease modifying therapy (DMT) and employment status. Patients who accepted DMTs in AusLong study were mostly administered with interferon-beta (88%), so DMTs use was code as yes/no in the analysis. When adjustment for the longitudinal use of DMTs, no significant difference with the primary analysis was seen (data not shown). So we used baseline DMTs in the primary analysis.

Due to the insignificant association between history of smoking, immune response to Epstein-Barr virus, levels of vitamin D, past exposure to ultraviolet radiation and *HLA-DR 15* genotype (data not shown), these factor were not adjusted in the multivariable model.

7.4.5 Data analysis

The effect of SLE and other covariates on time-to-conversion and time-to-relapse was calculated using Cox proportional hazards models, the latter for repeated events where multiple relapses by the same person are treated as independent observations but accounted for at the intra-individual level, and the time until a prior event dose not influence the composition of the risk set for a subsequent event. All variables of interest satisfied the proportional hazard assumption.

For conversion to MS analysis, the starting point is the date of FDE, and for relapse analysis, the starting point was the date of the event that brought the cases into the study[19]. Based on all available SLE data, we calculated the SLE variables 12 months and 6 months prior to each review in order to analyse its association with the subsequent occurrence of disease activity.

Predictors of annualised change in EDSS were evaluated using linear regression, adjusted for whether persons were having a relapse at the time of their 5-year review EDSS assessment. Because the annualised change in disability was highly skewed and for some cases the change of EDSS was zero, a log-transformation of the original score plus one was applied to satisfy linear regression assumptions of minimal heteroskedasticity. All means and coefficients, however, are back-transformed (for the reference group, minus 1 was performed) and presented on the original scale of the change in EDSS variable.

It is acknowledged that the increase of EDSS is not linear, however, the AusLong study participants were people with a first clinical diagnosis of demyelination at study entry and 87.6% had an EDSS score ≤ 4 at the 5-year review. Previous research has suggested that the disability progression is comparative linear when EDSS was ≤ 4. Time to particular EDSS hallmarks were less suitable as the participants were not assessed frequently enough by neurologists to reliably determine the date participants reached an EDSS of three or four. Therefore, annualised change of EDSS in the AusLong study was the best measure of disability progression.

Associations of other covariates (sex, age, employments status and baseline DMT) and annualised SLEs were analysed with linear regression.

7.5 Results

7.5.1 Characteristics of the cohort

A total of 251 cases with SLE data and clinical follow-up data were included in this analysis, 76.9% were females and the mean age at study entry was 38.8 years. At the 5-year review, 236 cases were still retained in the study. Among all cases, 18 were diagnosed as primary-progressive MS (PPMS), 180 as RRMS and 53 had not converted to MS at their final review. Therefore (198/251 = 78.9%) were diagnosed as MS before the 5th year review. A total of 433 relapses occurred during follow-up. On average each participant experienced 0.57 negative SLEs and 0.76 positive SLEs per year. Other characteristics of the cohort are shown in Table 1.

Table 7.1 Demographic and clinical characteristics of the cohort in the analysis

	Total cohort n (%)
Total	251
Female	193 (76.9)
Study site	
QLD	75 (29.88)
NSW	35 (13.94)
VIC	65 (25.9)
TAS	76 (30.28)
MS course at study entry	
CIS/RRMS	233 (92.83)
PPMS	18 (7.17)
Conversion during study	198 (78.88)
Relapses during study	433
Employed at baseline	204 (82.26)
Used DMT at baseline	53 (21.12)
	Mean (SD; Range)
Annualised SLE numbers	1.22 (0.83; 0, 4.81)
Annualised SLE numbers (positive)	0.57 (0.49; 0, 3.28)

Annualised SLE numbers (negative)	0.76 (0.62; 0, 3.26)
Annualised perceived SLE intensity	4.11 (3; 0, 15.97)
Annualised perceived SLE intensity (positive)	2.05 (1.71; 0, 9.44)
Annualised perceived SLE intensity (negative)	2.34 (2.11; 0, 9.94)
Annualised SLE load	41.19 (28.72; 0.87, 155.08)
Annualised SLE load (positive)	15.24 (11.33; 0.87, 69.77)
Annualised SLE load (negative)	31.86 (25.53; 0.87, 151.98)
Annualised SLE duration (days)	54.99 (66.18; 1.89, 479.01)
Annualised SLE duration (positive, days)	22.15 (22.57; 1.09, 206.75)
Annualised SLE duration (negative, days)	40.26 (62.95; 1.89, 463.96)
Age at study entry, years	38.76 (9.79; 18, 58)
MS duration from first symptoms to 5th year review, years	6.42 (2.38; 2.33, 21.2)
	Median (IQR)
EDSS at 5th year study review	2.00 (1.50, 2.75)
Annualised change of EDSS	0.27 (0.18; 0.41)

7.5.2 Association between baseline covariates and SLEs

Older age was associated with the reduced perceived positive SLEs (number, perceived severity, load and duration, $p<0.001$) while no association was found between age and perceived negative SLEs. Higher BMI at baseline was associated with a greater number of perceived negative SLEs, while there was no association with positive SLEs. There were no associations between sex, employment status or study centre and SLEs variables.

7.5.3 Association between SLE and Hazard of Conversion to MS

249 and 251 cases were included in the analysis of a risk window of 12 months and 6 months, respectively. Total SLE number, load, perceived severity and duration were not associated with the hazard of conversion to MS for both the 12 month and 6 month risk windows (Table 2). While some positive SLE variables had a dose-dependent association with the rate of MS conversion, they did not reach statistical significance. When we restricted our analysis to *major* positive SLEs, there was still

no association between positive SLEs and risk of conversion (risk window of 12 months and 6 months: test for trend p=0.49 & p=0.31, respectively). None of the negative SLE variables were associated with MS conversion, even when we restricted to *major* negative SLEs (risk window of 12 months and 6 months: test for trend p=0.82 & p=0.96, respectively).

Table 7.2 Associations between stressful life events and conversion to MS and relapses

	Conversion to MS [a]				**Relapse** [a]			
	12 months as risk window		6 months as risk window		12 months as risk window		6 months as risk window	
	aHR (95% CI)	p	aHR (95% CI)	p	aHR (95% CI)	p	aHR (95% CI)	p
SLE number prior to conversion/relapse								
Total								
0	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
1	1.35 (0.94, 1.93)	0.102	1.13 (0.76, 1.68)	0.544	0.93 (0.70, 1.22)	0.596	1.00 (0.77, 1.31)	0.978
2	1.19 (0.80, 1.78)	0.393	0.91 (0.54, 1.54)	0.733	0.90 (0.69, 1.19)	0.463	0.79 (0.56, 1.13)	0.196
≥3	0.92 (0.60, 1.41)	0.705	0.62 (0.29, 1.33)	0.216	0.84 (0.63, 1.11)	0.224	*0.60 (0.38, 0.94)*	*0.027*
Trend		0.910		0.358		0.224		*0.028*
Positive SLEs								
0	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
1	0.94 (0.65, 1.36)	0.755	0.85 (0.58, 1.27)	0.436	0.83 (0.64, 1.09)	0.179	0.88 (0.66, 1.18)	0.407
≥2	0.82 (0.55, 1.23)	0.341	0.69 (0.35, 1.36)	0.279	0.77 (0.57, 1.03)	0.079	0.64 (0.40, 1.02)	0.060
Trend		0.347		0.194		*0.035*		0.054
Negative SLEs								
0	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
1	*1.60 (1.13, 2.25)*	*0.008*	1.03 (0.66, 1.61)	0.894	1.06 (0.82, 1.35)	0.668	1.03 (0.80, 1.33)	0.796
≥2	1.18 (0.83, 1.67)	0.367	1.03 (0.61, 1.72)	0.925	1.10 (0.83, 1.46)	0.517	0.74 (0.50, 1.11)	0.147
Trend		0.133		0.888		0.493		0.277
Perceived SLE intensity prior to conversion/relapse								
Total								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-4	1.42 (0.97, 2.09)	0.074	1.25 (0.81, 1.95)	0.317	0.97 (0.73, 1.29)	0.856	1.01 (0.75, 1.35)	0.960
5-7	1.25 (0.86, 1.81)	0.248	0.89 (0.58, 1.37)	0.599	0.88 (0.64, 1.21)	0.427	0.85 (0.63, 1.16)	0.316
≥8	0.92 (0.61, 1.39)	0.703	0.72 (0.38, 1.34)	0.298	0.83 (0.63, 1.09)	0.185	*0.69 (0.48, 0.99)*	*0.047*

Trend		0.945		0.385		0.168		*0.053*
Positive SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-4	1.16 (0.79, 1.71)	0.456	0.95 (0.60, 1.51)	0.837	0.99 (0.76, 1.30)	0.953	0.95 (0.66, 1.36)	0.778
5-7	0.78 (0.50, 1.22)	0.283	0.69 (0.41, 1.17)	0.166	*0.67 (0.50, 0.90)*	*0.007*	*0.70 (0.51, 0.98)*	*0.035*
Trend		0.394		0.187		*0.010*		0.055
Negative SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-4	1.53 (1.08, 2.18)	0.018	0.85 (0.51, 1.41)	0.536	0.97 (0.74, 1.27)	0.833	0.94 (0.70, 1.26)	0.662
5-7	1.13 (0.79, 1.63)	0.495	1.17 (0.76, 1.80)	0.484	0.97 (0.74, 1.27)	0.834	0.86 (0.62, 1.19)	0.358
Trend		0.291		0.701		0.817		0.340
SLE load prior to conversion/relapse								
Total								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-40	1.18 (0.81, 1.72)	0.391	1.26 (0.86, 1.86)	0.239	0.96 (0.72, 1.29)	0.807	1.16 (0.85, 1.59)	0.349
>40-65	1.62 (1.04, 2.51)	0.033	0.96 (0.54, 1.71)	0.897	0.94 (0.70, 1.26)	0.686	0.82 (0.60, 1.13)	0.223
>65-100	1.09 (0.68, 1.74)	0.715	0.57 (0.26, 1.27)	0.167	0.86 (0.60, 1.22)	0.394	0.70 (0.46, 1.09)	0.115
>100	0.86 (0.54, 1.39)	0.548	0.83 (0.40, 1.71)	0.612	0.82 (0.59, 1.15)	0.255	0.63 (0.37, 1.06)	0.082
Trend		0.897		0.321		0.208		*0.021*
Positive SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-40	1.21 (0.73, 2.00)	0.470	1.14 (0.68, 1.90)	0.626	0.91 (0.60, 1.38)	0.643	1.04 (0.69, 1.57)	0.860
>40-65	0.95 (0.53, 1.69)	0.850	0.72 (0.38, 1.38)	0.322	0.72 (0.46, 1.12)	0.145	0.74 (0.44, 1.24)	0.256
>65-100	0.96 (0.60, 1.53)	0.856	0.84 (0.43, 1.63)	0.598	0.98 (0.71, 1.35)	0.910	0.91 (0.60, 1.39)	0.665
>100	0.68 (0.38, 1.21)	0.189	0.54 (0.22, 1.34)	0.187	*0.63 (0.40, 0.99)*	*0.044*	*0.46 (0.23, 0.91)*	*0.026*
Trend		0.289		0.139		0.052		*0.036*
Negative SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	

0-50	*1.58 (1.06, 2.37)*	*0.026*	1.11 (0.67, 1.84)	0.672	1.04 (0.79, 1.35)	0.799	1.06 (0.81, 1.39)	0.659
>50-95	*1.43 (1.01, 2.01)*	*0.042*	0.88 (0.50, 1.56)	0.671	1.03 (0.76, 1.39)	0.841	0.72 (0.47, 1.08)	0.110
>95	0.72 (0.40, 1.30)	0.277	1.02 (0.49, 2.13)	0.962	0.82 (0.56, 1.21)	0.317	0.74 (0.43, 1.28)	0.284
Trend		0.725		0.899		0.503		0.150
SLE duration prior to events								
Total								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-31 days	1.37 (0.96, 1.95)	0.087	1.14 (0.77, 1.68)	0.527	0.94 (0.71, 1.24)	0.655	1.01 (0.77, 1.32)	0.937
32-62 days	1.12 (0.74, 1.71)	0.584	0.80 (0.45, 1.40)	0.430	0.91 (0.69, 1.19)	0.485	0.75 (0.52, 1.07)	0.115
63-93 days	1.49 (0.90, 2.46)	0.122	0.51 (0.18, 1.47)	0.212	0.86 (0.61, 1.20)	0.373	*0.53 (0.30, 0.91)*	*0.022*
≥94 days	0.74 (0.43, 1.27)	0.271	1.15 (0.50, 2.62)	0.747	0.80 (0.55, 1.18)	0.263	0.87 (0.51, 1.48)	0.609
Trend		0.809		0.510		0.196		*0.044*
Positive SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-31 days	1.06 (0.71, 1.59)	0.761	0.93 (0.61, 1.40)	0.712	0.86 (0.65, 1.15)	0.313	0.90 (0.67, 1.22)	0.505
≥32 days	0.81 (0.52, 1.24)	0.334	0.62 (0.31, 1.23)	0.171	0.73 (0.53, 1.01)	0.057	0.62 (0.38, 1.00)	0.051
Trend		0.450		0.178		*0.042*		0.060
Negative SLEs								
No events	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
0-31 days	*1.53 (1.07, 2.18)*	*0.020*	1.03 (0.66, 1.62)	0.898	0.97 (0.75, 1.25)	0.806	1.01 (0.78, 1.31)	0.937
≥32 days	1.10 (0.77, 1.58)	0.605	0.96 (0.56, 1.66)	0.891	0.97 (0.73, 1.30)	0.860	0.69 (0.47, 1.02)	0.062
Trend		0.317		0.954		0.837		0.142

Fonts in bold and italic denoted statistically significant association

Adjusted for age, sex, study centres, BMI, baseline employment status and baseline DMTs

7.5.4 Association between SLE and Hazard of Relapses

Including 233 bout onset cases, we found that, in line with the previous conversion to MS analysis, *total* SLE number, load, perceived severity and duration were not associated with hazard of relapses for both the 12 month and 6 month risk windows (Table 2). However, we found that higher *positive* SLE variables in the previous 12 months were consistently associated with a lower hazard of relapse, including SLE number, perceived severity, SLE load, and SLE duration. In contrast, there were no associations for *negative* SLE variables including for major negative SLE number (risk window of 12 months and 6 months: p=0.561 & p=0.154, respectively). The magnitude of effect strengthened when restricting to a risk window of 6 months, although the associations became borderline significant due to a lower number of SLE events. All associations were adjusted for age, sex, BMI, baseline employment status and baseline DMT use Enhancement of the magnitude after adjustment was mostly driven by the negative confounder age (Supplementary Table 2).

7.5.5 Association between SLE and Annualised disability progression

We found no association between any of the SLE variables, including positive or negative SLEs (number, perceived severity, load and duration) and annualised change of EDSS (Table 3).

Table 7.3 Associations of baseline and longitudinal SLE variables and annualised change of EDSS

		Model [a]		Model [b]	
	No. (%)	**β (95% CI)**	**p**	**β (95% CI)**	**p**
SLE numbers					
Total					
0 - 3	31 (17.32)	+0.33 (0.25, 0.42)[c]		+0.34 (0.26, 0.42) [c]	

4 - 5	54 (30.17)	-0.07 (-0.18, 0.03)	0.167	-0.08 (-0.19, 0.02)	0.105
6 - 8	43 (24.02)	-0.01 (-0.12, 0.10)	0.823	-0.02 (-0.13, 0.09)	0.711
≥9	51 (28.49)	-0.03 (-0.14, 0.07)	0.528	-0.03 (-0.14, 0.07)	0.551
Trend			0.998		0.898
Positive SLEs					
0	25 (13.97)	+0.3 (0.21, 0.40) [c]		+0.32 (0.23, 0.41) [c]	
1 - 2	57 (31.84)	-0.01 (-0.12, 0.10)	0.829	-0.04 (-0.15, 0.07)	0.497
3 - 4	51 (28.49)	+0.02 (-0.10, 0.13)	0.786	-0.02 (-0.13, 0.09)	0.711
≥5	46 (25.70)	-0.02 (-0.13, 0.10)	0.769	+0.01 (-0.11, 0.13)	0.846
Trend			0.917		0.624
Negative SLEs					
0 - 1	42 (23.46)	+0.32 (0.24, 0.39) [c]		+0.32 (0.25, 0.39) [c]	
2	29 (16.20)	-0.04 (-0.15, 0.07)	0.526	-0.04 (-0.14, 0.07)	0.526
3 - 5	61 (34.08)	-0.04 (-0.13, 0.06)	0.448	-0.03 (-0.12, 0.06)	0.514
≥6	47 (26.26)	+0.01 (-0.09, 0.11)	0.882	0.00 (-0.10, 0.10)	0.954
Trend			0.916		0.968
Perceived SLE intensity					
Total					
0-9	40 (22.35)	+0.30 (0.23, 0.38) [c]		0.31 (0.24, 0.39) [c]	
10-19	48 (26.82)	-0.01 (-0.11, 0.08)	0.764	-0.03 (-0.13, 0.07)	0.575
20-32	45 (25.14)	+0.01 (-0.09, 0.11)	0.816	-0.01 (-0.11, 0.09)	0.862
≥33	46 (25.70)	-0.02 (-0.11, 0.08)	0.763	0.00 (-0.11, 0.10)	0.935
Trend			0.909		0.915
Positive SLEs					
0 - 4	39 (28.47)	+0.34 (0.26, 0.42) [c]		+0.33 (0.25, 0.41) [c]	
5 - 7	26 (18.98)	-0.08 (-0.2, 0.04)	0.217	-0.05 (-0.18, 0.07)	0.385
8 - 14	38 (27.74)	-0.01 (-0.12, 0.10)	0.831	-0.01 (-0.13, 0.10)	0.803
≥15	34 (24.82)	-0.03 (-0.14, 0.09)	0.646	0.00 (-0.12, 0.12)	0.988
Trend			0.845		0.917
Negative SLEs					
0 - 4	53 (32.72)	+0.32 (0.26, 0.38) [c]		+0.33 (0.27, 0.39) [c]	
5 - 7	26 (16.05)	-0.06 (-0.17, 0.05)	0.272	-0.07 (-0.17, 0.04)	0.212
8 - 14	41 (25.31)	-0.04 (-0.14, 0.06)	0.410	-0.05 (-0.15, 0.04)	0.287
≥15	42 (25.93)	+0.01 (-0.08, 0.11)	0.799	-0.00 (-0.10, 0.09)	0.974
Trend			0.864		0.914
SLE load					
Total					
0 - 110	44 (24.58)	+0.30 (0.23, 0.37) [c]		+0.30 (0.24, 0.37) [c]	
111 - 192	45 (25.14)	-0.04 (-0.13, 0.06)	0.421	-0.03 (-0.13, 0.06)	0.505
193 - 300	45 (25.14)	+0.03 (-0.07, 0.13)	0.513	+0.03 (-0.07, 0.13)	0.565
≥301	45 (25.14)	-0.01 (-0.10, 0.09)	0.904	-0.01 (-0.10, 0.09)	0.914
Trend			0.726		0.752
Positive SLEs					
0 - 44	35 (22.29)	+0.31 (0.23, 0.39) [c]		+0.32 (0.24, 0.40) [c]	
45 - 80	42 (26.75)	-0.01 (-0.12, 0.09)	0.799	-0.04 (-0.14, 0.07)	0.469

81 - 124	40 (25.48)	+0.01 (-0.10, 0.12)	0.840	-0.01 (-0.12, 0.10)	0.856
≥125	40 (25.48)	-0.02 (-0.13, 0.08)	0.657	+0.01 (-0.10, 0.12)	0.829
Trend			0.777		0.686
Negative SLEs					
0 - 70	42 (24.56)	+0.32 (0.25, 0.39) [c]		+0.32 (0.25, 0.40) [c]	
71 - 140	43 (25.15)	-0.04 (-0.14, 0.06)	0.453	-0.04 (-0.13, 0.06)	0.475
141 - 215	43 (25.15)	-0.03 (-0.13, 0.07)	0.579	-0.02 (-0.12, 0.08)	0.655
≥216	43 (25.15)	+0.01 (-0.09, 0.11)	0.863	-0.01 (-0.11, 0.09)	0.863
Trend			0.809		0.939
SLE duration					
Total					
0 - 123 days	32 (17.88)	+0.33 (0.25, 0.41) [c]		+0.34 (0.26, 0.42) [c]	
124 - 216 days	57 (31.84)	-0.08 (-0.18, 0.02)	0.133	-0.10 (-0.19, 0.00)	0.055
217 - 338 days	45 (25.14)	-0.02 (-0.12, 0.09)	0.746	+0.00 (-0.10, 0.11)	0.982
≥339 days	45 (25.14)	-0.00 (-0.11, 0.10)	0.954	+0.00 (-0.10, 0.11)	0.982
Trend			0.538		0.383
Positive SLEs					
0 - 61 days	31 (19.75)	0.34 (0.25, 0.42) [c]		0.34 (0.25, 0.42) [c]	
62 - 92 days	32 (20.38)	-0.05 (-0.17, 0.07)	0.425	-0.07 (-0.19, 0.04)	0.204
93 - 170 days	45 (28.66)	-0.03 (-0.14, 0.08)	0.564	-0.06 (-0.17, 0.05)	0.307
≥171 days	49 (31.21)	-0.05 (-0.16, 0.06)	0.382	+0.01 (-0.11, 0.12)	0.904
Trend			0.475		0.767
Negative SLEs					
0 - 61 days	36 (21.05)	+0.35 (0.27, 0.43) [c]		+0.35 (0.27, 0.43) [c]	
62 - 100 days	29 (16.96)	-0.08 (-0.19, 0.04)	0.190	-0.08 (-0.19, 0.03)	0.160
101 - 204 days	63 (36.84)	-0.08 (-0.17, 0.02)	0.131	-0.08 (-0.18, 0.02)	0.121
≥205 days	43 (25.15)	-0.00 (-0.11, 0.10)	0.941	-0.01 (-0.12, 0.09)	0.798
Trend			0.928		0.796

Fonts in bold and italic denoted statistically significant association
a: adjusted for relapse or not at 5th year review; b: further adjusted for age, sex, study centres, employment status and relapse numbers.
c: mean annualised change of EDSS in reference group

7.6 Discussion

In this prospective cohort study, we examined the association of SLEs and MS clinical course. We found that greater number, higher perceived severity and load and a longer duration of the events that were perceived as *positive* were consistently associated with a reduced hazard of relapses in a dose-dependent manner. Associations were less strong for conversion to MS. We found no associations

between *negative* SLEs and MS conversion or relapse risk. Neither positive nor negative SLEs were associated with disability progression.

Previous research has largely focused on the effects of negative events on MS clinical course, only two studies evaluated the relationship of positive SLE with MS, demonstrating some evidence of a beneficial impact on clinical course[4 11]. The associations were less strong for MS conversion, which may simply reflect the lower number of events in conversion analysis. Potential mechanisms underlying these associations could be through direct effects on the neuroendocrine system and immune responses, and an indirect effect via healthy behaviours and positive coping strategies (reviewed by Avvenuti and colleagues[20]). Our results indicated that events such as "gaining a new family member (new baby born or parent remarried)" or "you had an outstanding personal achievement (awards, grades, etc)" were factors that were experienced as predominantly positive. However, the effect may extend to smaller positive events such as weekend outings and social activity, which were not captured in our questionnaire. Indeed, positive psychological well-being has been associated with a decreasing hazard of mortality in a healthy (n=36,598; HR 0.82, $p<0.001$) as well as diseased populations (n=15,711; HR 0.98, $p=0.030$)[21]. A 2014 systematic review[22] on intervention techniques to decrease stress in people with MS, including Cognitive Behaviours Therapy (CBT) and relaxation training, found support that stress-management interventions may help people with MS reduce perceived stress, improve quality of life, reduce mental health comorbidities, and improve outcomes associated with the disease. Our evidence warrants that these interventions could include a focus on the benefits of positive events.

The absence of an effect of negative SLEs on disease outcomes was unexpected in this study as a number of previous studies have found significant associations between negative SLEs and adverse MS clinical course[5 23 24]. Even limiting to *major* negative SLEs (loss of family/friend or physical injury), we found no association with any clinical outcomes. Different methodologies for classifying the valence of SLE could account for the disparities in findings across studies. Buljevac and colleagues[5] only asked about perceived emotionally stressful events and asked patients to keep diaries. Two neurologists excluded diary events that were deemed to be directly connected to multiple sclerosis symptomatology. While Potagas and colleagues[23] only looked at anxiety provoking stressful event and did not ask participants if they believe the event to be negative only if was stressful – an event can be stressful but ultimately viewed by the person as helpful. There are some other studies that are consistent with our findings. One study following 36 MS patients up to 100 weeks[12] found that major negative SLEs were not associated with the emergence of new enhancing lesion on MRI. Some other research[25-27]assessing the association of occurrence of negative stressors with MS onset has typically found no significant associations. Nisipeanu and colleagues actually demonstrated a beneficial impact of ambient negative stress on clinical course[28], following 32 MS patients for two months in Israel during the 1991 Persian Gulf War when patients were under threat of missile attacks and finding that relapse rates were significantly lower during that period compared to the previous two year period ($p<0.001$). However, not controlling for potential confounders especially the treatment effects and small sample size influenced the validity of this study.

We found no evidence to support an association between SLEs and annualised change of EDSS. This is consistent with another prospective study[12] that found that SLEs were not associated with disability progression as measured by EDSS ($p>0.05$). These results conflict with that found by Brown and colleagues, which[4] even showed that acute stressors were associated with a slower progression of disability. With a cross-sectional study design, our group recently found a positive association between higher psychological dysfunctions (fatigue, depression and anxiety) and higher EDSS score[2].

In this study, a shorter risk window (6 months compared to 12 months) was associated with a stronger effect on subsequent relapse, suggesting the effect of positive SLEs occurs biologically within months. A number of other studies modeled the lag between stressful life events and MS onset or relapse. One study[23] found that after the emergence of SLEs, the risk of first and second relapse was increased ($p<0.001$), while there was no association with the third relapse ($p=0.21$). Similarly, a randomized controlled trial[7] showed a stress management intervention was associated with decreased disease activity (measured as emergence of lesions in MRI), but these effects were not sustained beyond the intervention (24 weeks).

A strength of this study is the prospective longitudinal design allowing the analysis where the SLEs occurred prior to the outcome (conversion to MS/relapses), therefore establishing the correct temporal relationship. Also, the method of reporting of SLEs was identical in all participants, using a form-based method rather than free-text response, therefore ensuring systematic measurement and reducing the risk of recall bias. Internal consistency is another strength in this study, as different but complementary SLE measures (number, perceived load, load and duration) found

agreeable results. Moreover, using two different risk windows found evidence of a stronger association when the interval between exposure and outcome was shorter, substantiating the validity of therelationship.

Participants in AusLong study were recruited from relatively contemporary populations with a standardised protocol, therefore, birth cohort effect had a relatively less influence on the results. However, we still performed a sensitivity analysis based on the date of birth of the cases (1947-1969, 1961-1970, 1971-1988), and no significant interaction between SLEs variables and date of birth were found.

One limitation of this study was not having measured potential confounders such as SLEs during childhood, which could be germane in MS onset. That said, our demonstration of a stronger association in closer temporal proximity to clinical outcomes may mean that these historical events may be less impactful than those measured later in life. Another limitation of the study is that we did not measure psychological dysfunctions (depression, fatigue and anxiety) until 5-year review, which can relatively predict negative physical and mental health outcomes. Similarly, we did not assess positive psychological states. SLEs were recorded retrospectively at annual review, so it was difficult to make sure every SLEs recorded was accurate (especially for SLEs recorded at 2/3-year review, which included SLEs happened from one year prior to FCD to 2/3-year review). Some patients might have the idea that stressors were associated with disease activity, which was able to result in the recall bias. We therefore performed another analysis to examine the role of relapse in SLEs, and no association between relapse and SLEs was seen ($p=0.93$). We could not

make sure every SLEs recorded were accurate, however, no evidence was seen that the potential misclassification of SLEs was recorded differential by relapses.

In conclusion, we found consistent evidence that positive SLEs were associated with a reduced hazard of relapses, suggesting that positive SLEs and possibly an overall positive psychological well-being may be beneficial in MS clinical course.

7.7 Postscript

This chapter has provided some data on the experienced stressful life events in MS patients and their associations with disease activity and disability progression. The next chapter draws the conclusions from the previous studies and plans of the future research will be described.

7.8 Reference

1. Wood B, van der Mei IA, Ponsonby AL, et al. Prevalence and concurrence of anxiety, depression and fatigue over time in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2013;19(2):217-24. doi: 10.1177/1352458512450351 [published Online First: 2012/06/26]
2. Simpson S, Jr., Tan H, Otahal P, et al. Anxiety, depression and fatigue at 5-year review following CNS demyelination. *Acta neurologica Scandinavica* 2016 doi: 10.1111/ane.12554 [published Online First: 2016/01/13]
3. Mohr DC, Hart SL, Julian L, et al. Association between stressful life events and exacerbation in multiple sclerosis: a meta-analysis. *Bmj* 2004;328(7442):731. doi: 10.1136/bmj.38041.724421.55
4. Brown RF, Tennant CC, Sharrock M, et al. Relationship between stress and relapse in multiple sclerosis: Part I. Important features. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2006;12(4):453-64. [published Online First: 2006/08/12]
5. Buljevac D, Hop WC, Reedeker W, et al. Self reported stressful life events and exacerbations in multiple sclerosis: prospective study. *Bmj* 2003;327(7416):646. doi: 10.1136/bmj.327.7416.646
6. Artemiadis AK, Anagnostouli MC, Alexopoulos EC. Stress as a risk factor for multiple sclerosis onset or relapse: a systematic review. *Neuroepidemiology* 2011;36(2):109-20. doi: 10.1159/000323953
7. Mohr DC, Lovera J, Brown T, et al. A randomized trial of stress management for the prevention of new brain lesions in MS. *Neurology* 2012;79(5):412-9. doi:

10.1212/WNL.0b013e3182616ff9 [published Online First: 2012/07/13]
8. Somer E, Golan D, Dishon S, et al. Patients with multiple sclerosis in a war zone: coping strategies associated with reduced risk for relapse. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(4):463-71. doi: 10.1177/1352458509358714 [published Online First: 2010/01/21]
9. Pagnini F, Bosma CM, Phillips D, et al. Symptom changes in multiple sclerosis following psychological interventions: a systematic review. *BMC neurology* 2014;14:222. doi: 10.1186/s12883-014-0222-z [published Online First: 2014/12/01]
10. Strober LB, Arnett PA. Stressful life events precede exacerbations of multiple sclerosis. *Psychology, health & medicine* 2015:1-9. doi: 10.1080/13548506.2015.1093645 [published Online First: 2015/10/13]
11. Burns MN, Nawacki E, Kwasny MJ, et al. Do positive or negative stressful events predict the development of new brain lesions in people with multiple sclerosis? *Psychological medicine* 2014;44(2):349-59. doi: 10.1017/s0033291713000755 [published Online First: 2013/05/18]
12. Mohr DC, Goodkin DE, Bacchetti P, et al. Psychological stress and the subsequent appearance of new brain MRI lesions in MS. *Neurology* 2000;55(1):55-61. [published Online First: 2000/07/13]
13. Lucas R, Ponsonby AL, McMichael A, et al. Observational analytic studies in multiple sclerosis: controlling bias through study design and conduct. The Australian Multicentre Study of Environment and Immune Function. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2007;13(7):827-39. doi: 10.1177/1352458507077174 [published Online First: 2007/09/21]
14. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Annals of neurology* 2011;69(2):292-302. doi: 10.1002/ana.22366
15. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Annals of neurology* 2001;50(1):121-7.
16. Holmes TH, Rahe RH. The Social Readjustment Rating Scale. *Journal of psychosomatic research* 1967;11(2):213-8.
17. Scully JA, Tosi H, Banning K. Life Event Checklists: Revisiting the Social Readjustment Rating Scale after 30 Years. *Educational and Psychological Measurement* 2000;60(6):864-76. doi: 10.1177/00131640021970952
18. Tettey P, Simpson S, Taylor B, et al. An adverse lipid profile and increased levels of adiposity significantly predict clinical course after a first demyelinating event. *Journal of neurology, neurosurgery, and psychiatry* 2017;88(5):395-401. doi: 10.1136/jnnp-2016-315037 [published Online First: 2017/03/23]
19. Pan G, Simpson S, Jr., van der Mei I, et al. Role of genetic susceptibility variants in predicting clinical course in multiple sclerosis: a cohort study. *Journal of neurology, neurosurgery, and psychiatry* 2016;87(11):1204-11. doi: 10.1136/jnnp-2016-313722 [published Online First: 2016/08/26]
20. Avvenuti G, Baiardini I, Giardini A. Optimism's Explicative Role for Chronic Diseases. *Frontiers in psychology* 2016;7:295. doi: 10.3389/fpsyg.2016.00295 [published Online First: 2016/03/15]
21. Chida Y, Steptoe A. Positive psychological well-being and mortality: a

quantitative review of prospective observational studies. *Psychosomatic medicine* 2008;70(7):741-56. doi: 10.1097/PSY.0b013e31818105ba [published Online First: 2008/08/30]
22. Reynard AK, Sullivan AB, Rae-Grant A. A systematic review of stress-management interventions for multiple sclerosis patients. *International journal of MS care* 2014;16(3):140-4. doi: 10.7224/1537-2073.2013-034 [published Online First: 2014/10/23]
23. Potagas C, Mitsonis C, Watier L, et al. Influence of anxiety and reported stressful life events on relapses in multiple sclerosis: a prospective study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(9):1262-8. doi: 10.1177/1352458508095331 [published Online First: 2008/08/30]
24. Mohr DC, Goodkin DE, Nelson S, et al. Moderating Effects of Coping on the Relationship Between Stress and the Development of New Brain Lesions in Multiple Sclerosis. *Psychosomatic medicine* 2002;64(5):803-9. doi: 10.1097/01.psy.0000024238.11538.ec
25. Nielsen NM, Pedersen BV, Stenager E, et al. Stressful life-events in childhood and risk of multiple sclerosis: a Danish nationwide cohort study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(12):1609-15. doi: 10.1177/1352458514528761 [published Online First: 2014/04/02]
26. Nielsen NM, Bager P, Simonsen J, et al. Major stressful life events in adulthood and risk of multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2014;85(10):1103-8. doi: 10.1136/jnnp-2013-307181 [published Online First: 2014/03/13]
27. Riise T, Mohr DC, Munger KL, et al. Stress and the risk of multiple sclerosis. *Neurology* 2011;76(22):1866-71. doi: 10.1212/WNL.0b013e31821d74c5 [published Online First: 2011/06/01]
28. Nisipeanu P, Korczyn AD. Psychological stress as risk factor for exacerbations in multiple sclerosis. *Neurology* 1993;43(7):1311-2.

7.9 Supplementary Tables

Table 7.4 Univariable associations of stressful stress life events and conversion to MS.

	12 months as risk window			6 months as risk window		
	failures/person-years (rate)	**HR (95% CI)**	**p**	**failures/person-years (rate)**	**HR (95% CI)**	**p**
SLE numbers prior to conversion						
Total						
0	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
1	39/108.88 (0.36)	1.26 (0.88, 1.79)	0.203	35/108.96 (0.32)	1.04 (0.71, 1.53)	0.841
2	28/98.93 (0.28)	1.03 (0.69, 1.53)	0.878	16/68.50 (0.23)	0.79 (0.46, 1.36)	0.395
≥3	24/115.45 (0.21)	0.83 (0.55, 1.27)	0.393	7/51.45 (0.14)	0.53 (0.25, 1.14)	0.104
Trend			0.558			0.107
Positive SLEs						
0	143/436.33 (0.33)	1.00 (reference)		166/515.61 (0.32)	1.00 (reference)	
1	31/131.47 (0.24)	0.79 (0.55, 1.13)	0.197	23/99.06 (0.23)	0.77 (0.51, 1.15)	0.206
≥2	22/95.5 (0.23)	0.85 (0.57, 1.27)	0.440	9/48.89 (0.18)	0.71 (0.35, 1.42)	0.331
Trend			0.240			0.151
Negative SLEs						
0	118/410.32 (0.29)	1.00 (reference)		156/498.11 (0.31)	1.00 (reference)	
1	42/113.84 (0.37)	1.51 (1.06, 2.14)	0.021	26/98.96 (0.26)	0.96 (0.61, 1.49)	0.846
≥2	36/139.14 (0.26)	1.06 (0.75, 1.50)	0.741	16/66.48 (0.24)	0.86 (0.52, 1.42)	0.547
Trend			0.371			0.550
Perceived SLE intensity prior to conversion						
Total						
No events	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-4	30/78.81 (0.38)	1.4 (0.96, 2.04)	0.080	24/67.23 (0.36)	1.26 (0.81, 1.96)	0.304

5-7		31/101.58 (0.31)	1.1 (0.76, 1.57)	0.621	24/102.22 (0.23)	0.76 (0.50, 1.17)	0.220
≥8		30/142.88 (0.21)	0.81 (0.54, 1.2)	0.292	10/59.45 (0.17)	0.61 (0.33, 1.14)	0.121
	Trend			0.461			0.092
Positive SLEs							
No events		105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-4		27/91.81 (0.29)	1.16 (0.79, 1.71)	0.456	17/63.98 (0.27)	0.93 (0.57, 1.50)	0.765
5-7		26/135.16 (0.19)	0.78 (0.50, 1.22)	0.283	15/83.96 (0.18)	0.61 (0.36, 1.02)	0.059
	Trend			0.394			0.067
Negative SLEs							
No events		105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-4		37/101 (0.37)	***1.50 (1.04, 2.15)***	***0.028***	18/84.43 (0.21)	0.80 (0.47, 1.34)	0.395
5-7		41/151.98 (0.27)	1.02 (0.73, 1.44)	0.904	24/81.01 (0.30)	0.98 (0.65, 1.49)	0.940
	Trend			0.619			0.727
SLE load prior to conversion							
Total							
No events		105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-40		26/76.90 (0.34)	1.13 (0.77, 1.65)	0.534	26/69.33 (0.38)	1.24 (0.84, 1.83)	0.277
>40-65		27/62.98 (0.43)	1.49 (0.99, 2.23)	0.054	18/63.91 (0.28)	0.91 (0.53, 1.57)	0.741
>65-100		19/86.54 (0.22)	0.87 (0.55, 1.37)	0.544	7/56.55 (0.12)	0.44 (0.20, 0.95)	0.036
>100-		19/96.84 (0.20)	0.79 (0.49, 1.28)	0.341	7/37.99 (0.18)	0.70 (0.34, 1.45)	0.340
	Trend			0.523			0.066
Positive SLEs							
No events		105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-40		16/57.07 (0.28)	1.01 (0.63, 1.63)	0.965	11/35.98 (0.31)	0.98 (0.59, 1.64)	0.951
>40-65		12/57.62 (0.21)	0.72 (0.41, 1.26)	0.254	8/45.85 (0.17)	0.58 (0.29, 1.16)	0.123
>65-100		15/57.89 (0.26)	0.97 (0.61, 1.54)	0.900	8/31.29 (0.26)	0.91 (0.43, 1.91)	0.795
>100-		10/54.39 (0.18)	0.72 (0.40, 1.29)	0.275	5/34.82 (0.14)	0.54 (0.22, 1.32)	0.177
	Trend			0.252			0.102

Negative SLEs						
No events	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-50	34/87.67 (0.39)	1.55 (1.03, 2.33)	0.038	21/75.06 (0.28)	1.12 (0.67, 1.87)	0.677
>50-95	32/89.02 (0.36)	1.29 (0.92, 1.80)	0.136	14/56.96 (0.25)	0.73 (0.42, 1.25)	0.252
>95-	12/76.29 (0.16)	0.66 (0.37, 1.19)	0.165	7/32.28 (0.22)	0.83 (0.40, 1.74)	0.624
Trend			0.855			0.354
SLE duration prior to conversion						
Total						
No events	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-31 days	40/109.62 (0.36)	1.27 (0.90, 1.81)	0.175	36/109.35 (0.33)	1.05 (0.72, 1.53)	0.804
32-62 days	24/92.25 (0.26)	1.00 (0.66, 1.52)	0.990	12/62.48 (0.19)	0.69 (0.38, 1.25)	0.217
63-93 days	13/40.08 (0.32)	1.22 (0.72, 2.09)	0.456	4/34.59 (0.12)	0.44 (0.16, 1.23)	0.118
≥94 days	14/81.32 (0.17)	0.67 (0.40, 1.13)	0.135	6/22.49 (0.27)	0.99 (0.43, 2.25)	0.974
Trend			0.353			0.183
Positive SLEs						
No events	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-31 days	31/127.81 (0.24)	0.88 (0.6, 1.28)	0.494	23/95.96 (0.24)	0.80 (0.53, 1.22)	0.303
≥32 days	22/99.16 (0.22)	0.83 (0.55, 1.27)	0.394	9/51.99 (0.17)	0.62 (0.31, 1.27)	0.194
Trend			0.336			0.114
Negative SLEs						
No events	105/340.03 (0.31)	1.00 (reference)		140/434.65 (0.32)	1.00 (reference)	
0-31 days	42/113.15 (0.37)	***1.45 (1.01, 2.08)***	***0.043***	27/99.02 (0.27)	0.96 (0.61, 1.49)	0.843
≥32 days	36/139.83 (0.26)	1.00 (0.70, 1.43)	0.993	15/66.42 (0.23)	0.81 (0.48, 1.36)	0.420
Trend			0.632			0.444

Fonts in bold and italic denoted statistically significant association

Table 7.5 Univariable associations of stressful stress life events and relapses.

	12 months as risk window			6 months as risk window		
	failures/person-years (rate)	HR (95% CI)	p	failures/person-years (rate)	HR (95% CI)	p
SLE numbers prior to relapse						
Total						
0	191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
1	98/319.54 (0.31)	0.97 (0.74, 1.27)	0.825	105/313.24 (0.34)	1.03 (0.79, 1.35)	0.831
2	65/216.23 (0.30)	0.92 (0.71, 1.20)	0.540	39/156.35 (0.25)	0.80 (0.57, 1.12)	0.185
≥3	79/284.86 (0.28)	0.89 (0.66, 1.21)	0.462	24/124.78 (0.19)	0.65 (0.41, 1.02)	0.063
Trend			0.424			0.054
Positive SLEs						
0	286/784.95 (0.36)	1.00 (reference)		339/967.83 (0.35)	1.00 (reference)	
1	88/327.08 (0.27)	0.83 (0.64, 1.09)	0.182	70/259.75 (0.27)	0.89 (0.67, 1.19)	0.440
≥2	59/224.24 (0.26)	0.84 (0.62, 1.14)	0.266	24/108.68 (0.22)	0.72 (0.45, 1.16)	0.177
Trend			0.136			0.139
Negative SLEs						
0	228/699.57 (0.33)	1.00 (reference)		308/908.45 (0.34)	1.00 (reference)	
1	105/329.06 (0.32)	1.13 (0.89, 1.45)	0.310	91/274.43 (0.33)	1.06 (0.83, 1.35)	0.657
≥2	100/307.64 (0.33)	1.08 (0.81, 1.45)	0.604	34/153.39 (0.22)	0.74 (0.51, 1.09)	0.130
Trend			0.504			0.279
Perceived SLE intensity prior to relapse						
Total						
No events	191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-4	88/276.00 (0.32)	1.01 (0.76, 1.35)	0.926	79/234.10 (0.34)	1.05 (0.78, 1.41)	0.742
5-7	60/209.93 (0.29)	0.88 (0.65, 1.20)	0.423	50/188.44 (0.27)	0.82 (0.61, 1.11)	0.206
8-	94/334.70 (0.28)	0.89 (0.67, 1.18)	0.427	39/171.84 (0.23)	0.75 (0.53, 1.07)	0.116
Trend			0.353			0.090

Positive SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-4		75/228.35 (0.33)	1.03 (0.78, 1.36)	0.841	48/164.69 (0.29)	0.97 (0.68, 1.39)	0.889
5-7		72/322.97 (0.22)	***0.72 (0.54, 0.97)***	***0.032***	46/203.74 (0.23)	0.74 (0.54, 1.02)	0.070
	Trend			***0.044***			0.104
Negative SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-4		105/320.28 (0.33)	1.05 (0.79, 1.40)	0.741	75/240.11 (0.31)	0.97 (0.72, 1.30)	0.830
5-7		100/316.41 (0.32)	1.00 (0.76, 1.30)	0.974	50/187.71 (0.27)	0.85 (0.63, 1.17)	0.324
	Trend			0.976			0.349
SLE load prior to relapse							
Total							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-40		62/209.50 (0.30)	0.94 (0.70, 1.26)	0.669	67/191.37 (0.35)	1.17 (0.86, 1.59)	0.333
>40-65		74/215.97 (0.34)	1.05 (0.79, 1.39)	0.751	61/195.95 (0.31)	0.88 (0.65, 1.20)	0.428
>65-100		48/173.60 (0.28)	0.88 (0.62, 1.25)	0.471	22/111.03 (0.20)	***0.64 (0.42, 0.98)***	***0.039***
>100-		58/215.69 (0.27)	0.87 (0.61, 1.23)	0.422	18/91.30 (0.20)	0.70 (0.41, 1.20)	0.194
	Trend			0.434			***0.042***
Positive SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-40		39/138.57 (0.28)	0.90 (0.62, 1.32)	0.604	33/98.10 (0.34)	1.03 (0.70, 1.54)	0.866
>40-65		28/122.85 (0.23)	0.72 (0.45, 1.14)	0.161	23/108.00 (0.21)	0.76 (0.45, 1.27)	0.299
>65-100		47/142.44 (0.33)	1.07 (0.78, 1.47)	0.673	27/91.30 (0.30)	0.95 (0.62, 1.46)	0.810
>100-		33/147.68 (0.22)	0.72 (0.45, 1.14)	0.161	11/70.21 (0.16)	0.53 (0.28, 1.04)	0.065
	Trend			0.219			0.088
Negative SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-50		96/272.95 (0.35)	1.14 (0.87, 1.49)	0.355	81/224.60 (0.36)	1.12 (0.85, 1.47)	0.435

>50-95		67/201.32 (0.33)	1.01 (0.76, 1.35)	0.928	28/126.45 (0.22)	0.67 (0.45, 1.00)	0.050
>95-		42/156.52 (0.27)	0.88 (0.58, 1.33)	0.534	16/72.87 (0.22)	0.79 (0.44, 1.41)	0.416
	Trend			0.677			0.155
SLE duration prior to relapse							
Total							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-31 days		98/314.27 (0.31)	0.98 (0.75, 1.29)	0.886	105/310.85 (0.34)	1.03 (0.79, 1.35)	0.818
32-62 days		60/206.62 (0.29)	0.93 (0.71, 1.21)	0.570	35/151.69 (0.23)	0.75 (0.53, 1.06)	0.104
63-93 days		38/132.21 (0.29)	0.93 (0.66, 1.32)	0.699	14/90.23 (0.16)	0.54 (0.31, 0.92)	0.025
≥94 days		46/167.54 (0.27)	0.84 (0.56, 1.26)	0.398	14/41.61 (0.34)	1.03 (0.61, 1.74)	0.914
	Trend			0.364			0.100
Positive SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-31 days		88/323.32 (0.27)	0.87 (0.66, 1.15)	0.336	70/257.02 (0.27)	0.91 (0.67, 1.22)	0.524
≥32 days		59/229.48 (0.26)	0.83 (0.60, 1.15)	0.261	24/111.41 (0.22)	0.70 (0.44, 1.14)	0.155
	Trend			0.195			0.145
Negative SLEs							
No events		191/515.63 (0.37)	1.00 (reference)		265/741.89 (0.36)	1.00 (reference)	
0-31 days		104/323.13 (0.32)	1.06 (0.82, 1.36)	0.668	91/271.71 (0.33)	1.03 (0.80, 1.34)	0.818
≥32 days		101/313.56 (0.32)	0.99 (0.74, 1.33)	0.947	34/156.11 (0.22)	0.71 (0.49, 1.04)	0.077
	Trend			0.998			0.176

Fonts in bold and italic denoted statistically significant association

Chapter 8: Summary and future directions

Treatments for relapsing MS patients advanced markedly during the last decade, but conversion to secondary-progressive stage could not be prevented for relapsing MS patients. Mechanisms underlying the disease progression and conversion to secondary-progressive stage are still illusive. Therapeutic options for progressive MS patients were limited; only one recent phase 3 RCT showed patients with ocrelizumab were associated with decreased rate of clinical progression[1]. However, subjects chosen in this study were comparatively benign and some patients still experienced periodical exacerbations. Therefore, preventing and treating MS from other perspectives is of great importance. Understanding what factors drive the progression of MS especially when these factors are modifiable may divulge these essential findings for people with MS.

Some large prospective cohort studies endeavoured to deal with these puzzles, but no consistent conclusion has been achieved. BENEFIT and MSL study[2 3] showed that higher levels of anti-EBNA IgG were not associated with subsequent conversion to MS and relapses, but results from some other good-quality studies[4 5] were not in line with this finding. Although a number of well-designed studies[6-8] found tobacco smoking could accelerate the progression of MS, some study[2] still showed a negative result. Relapse rate decreased significantly during pregnancy and increased in the first 3 months postpartum, but the long-term effects of pregnancy on MS clinical course is still less certain[9-12].

This thesis evaluated established and potential risk factors and their effects on ASO of MS, which assists in the evidence base for strategies that delay and prevent the onset of MS. The thesis also explored the associations between viral infections and SLEs on conversion to MS,

time to relapses, and disability progression, contributing to the understanding of the mechanisms underlying disease progression.

8.1 Summary of findings and implications

In Chapter 4, the first aim of the thesis was addressed using a unique and large international database, including 21 countries with 22162 diagnosed MS patients. We found a significant association between latitude of residence and ASO, and variation of UVR was the possible candidate underlying this latitudinal gradient. When compared to patients living in lower latitudes, the onset of symptoms was 1.9 years earlier among those residing at higher latitudes, and a similar relationship was found for ambient UVR. Our study also demonstrated that sex was associated with ASO, with female patients having on average a 5-month earlier onset than male patients. ASO in relapsing-onset patients was approximately 9 years earlier than that for progressive-onset patients. Optimising sun exposure and vitamin D during the life course was estimated to make an important impact at the population level on the prevention of MS[13]. Our findings seem to suggest that this may also delay the onset in patients with MS. Population health messages that counter the decrease of outdoor behaviour in our young generations will be important to prevent and possibly delay MS.

Chapter 5 addressed the second aim of the thesis. We examined the effects of detailed environmental/behavioural factors on ASO. We found that a history of tobacco smoking was associated with 3.05-years later ASO, whereas a history of marijuana use was associated with 2.80-years earlier ASO. In agreement with the first study in this thesis, progressive-onset cases had later ASO than relapsing onset cases, and an initial presentation of cerebral function impairment was associated with an approximately 4years later onset. This study found that sex, offspring number, latitude of study site, and UVR exposure were not associated with ASO, which does not neatly align with the first study. Small sample size and

less latitudinal spread may partly explain the difference. We also found that a history of infectious mononucleosis, *HLA-DR15* and *HLA-A2* genotypes, latitude, UVR, and serum vitamin D metabolite levels were not significantly associated with ASO. To examine whether the results were due to an age or cohort effect, we repeated the analysis in the matched healthy controls. Each control was given the ASO of the matched cases. If the association was also observed in controls, then that is likely due to an age or cohort effect. We found no association between smoking status and ASO (never smokers vs. ever smokers: adjusted β=+0.11 years, 95% CI -1.54 - 1.77, p=0.89), suggesting the significant association in cases was not due to an age or cohort effect. Few studies have examined factors associated with symptom onset, and the examination is challenged by cohort effects and inherent correlation exposures and the outcome measure. Therefore, additional studies are required to examine age of onset effects.

Chapter 5 reported the associations between EBV and HHV6 infections on the MS clinical course (conversion to MS, time to relapses, and annualised change of EDSS). Although a higher immune response to anti-EBNA IgG has been confirmed to contribute to the onset of MS, we found no association between levels of anti-EBNA IgG and MS clinical course, and other serological parameters such as EBV DNA, early antigen (diffuse and restricted), and viral capsid antigen were not associated with any measured MS clinical course. A history of infectious mononucleosis at baseline was associated with increased hazard of relapse, but not with conversion to MS, although the direction and magnitude were similar. No association was found between serological or viral load parameters of HHV6 and any clinical course outcome. Thus, while the evidence is overwhelming that EBV is causally related to the onset of MS, its role in the clinical course is far less certain[2].

In Chapter 6, we evaluated the effects of SLEs on MS clinical course. For conversion to MS and relapses analysis, we summarised SLE information 6 or 12 months prior to conversion to MS or relapse and evaluated its influence on subsequent disease activity. We found that a greater number, higher perceived severity and load, and a longer duration of the events that were perceived as positive were consistently associated with a reduced hazard of relapse in a dose-dependent manner. Associations were less strong for conversion to MS, however. There were no associations between perceived negative SLEs and MS conversion or relapse risk. These findings offer a positive new story to people with MS and can empower them to increase their focus on positive events. The findings are very suitable to be translated into practice and align with the beneficial health effects of interventions that reduce stress.

In summary, the findings from this thesis underline the importance of modifiable environmental or behavioural factors on delaying the age of disease onset and reducing the hazard of conversion to MS and relapses.

8.2 Future directions

Future directions as suggested by this thesis are to:

1. *Determine whether the other lifestyle factors such as smoking, physical activity, offspring number prior to onset, and alcohol and coffee consumption could influence the clinical course of MS.*

   In relation to the clinical course in patients with MS, there are other modifiable factors, such as tobacco smoking, physical activity, offspring number prior to onset, alcohol and coffee consumption, which have not been assessed in this thesis. The overall evidence base in relation to these variables and MS clinical course is relatively low[7-9 12 14-20]. This could assist in the

understanding of the mechanism underlying MS progression. Furthermore, our findings may help clinicians to provide some advice with direct and stronger evidence for considering adding these interventions as a strategy for prevention of disease progression in MS patients at the early stage (some therapeutic studies suggested that only interventions at the early stage can influence the long-term prognosis[21]).

2. *Determine association of effects between demographic factors and initial clinical symptoms on MS clinical course*

    a. *Examining whether absence/presence of some presentations at disease onset (pyramid, cerebellar, brainstem, sensory, bowel and bladder, cerebral and visual dysfunction) could influence MS clinical course*

    b. *Examining whether gender, latitude, and education levels could influence MS clinical course*

    Although current studies have confirmed the effects of some demographic factors such as latitude of residence and gender on MS onset[22], their associations with MS clinical course are still unclear. At baseline interview, demographic information and initial symptoms at FDE were recorded and confirmed by study neurologists. Clarifying the absence or presence of the association between demographic or initial symptoms at FDE and subsequent disease activity and disability progression could help the clinicians to predict the prognosis of MS patients from disease onset, and may assist in the clarifying of the pathological pathways influencing the progression of MS.

3. *Determine whether established modifiable risk factors (EBV infection, infectious mononucleosis, vitamin D levels, UVR, and smoking) could influence MS clinical course at the late stage (conversion from RRMS to SPMS)*

   This thesis did not discuss associations between some modifiable factors at the early stage and long-term MS clinical course. Current disease-modifying treatments are effective for relapsing-remitting patients, but there is a lack of treatment options when they convert to SPMS[23]. Therefore, preventing patients from converting to SPMS is a primary target for relapsing-onset patients. Our study could assist in the understanding of the mechanisms underlying the association between currently established risk factors and conversion from RRMS to SPMS, and preventing the disease progression via modifying behaviours.

4. *Determine whether frequency of relapses at the early stage could predict the disability progression and conversion to SPMS.*

   Current disease-modifying treatments are able to reduce the frequency of relapses for MS patients, but the association between relapse rate and long-term disability accrual is still uncertain. Although some treatment studies[24-29] demonstrated the positive relationship between relapse frequency and long-term disability progression, one prospective cohort study[30] suggested that only disease activity within 2 years after onset could influence the long-term disability progression and conversion to SPMS. With measurement of relapse frequency up to 10 years after disease onset, this study

may aid in the understanding of the predictable effects of early clinical factors on long-term disease.

8.3 References

1. Montalban X, Hauser SL, Kappos L, et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *The New England journal of medicine* 2016 doi: 10.1056/NEJMoa1606468 [published Online First: 2016/12/22]
2. Munger KL, Fitzgerald KC, Freedman MS, et al. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. *Neurology* 2015;85(19):1694-701. doi: 10.1212/wnl.0000000000002099 [published Online First: 2015/10/11]
3. Simpson S, Jr., Taylor B, Dwyer DE, et al. Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2012;18(6):799-806. doi: 10.1177/1352458511428081 [published Online First: 2011/11/16]
4. Lünemann JD, Tintoré M, Messmer B, et al. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Annals of neurology* 2010;67(2):159-69. doi: 10.1002/ana.21886
5. Makhani N, Banwell B, Tellier R, et al. Viral exposures and MS outcome in a prospective cohort of children with acquired demyelination. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(3):385-8. doi: 10.1177/1352458515595876 [published Online First: 2015/07/23]
6. Di Pauli F, Reindl M, Ehling R, et al. Smoking is a risk factor for early conversion to clinically definite multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2008;14(8):1026-30. doi: 10.1177/1352458508093679 [published Online First: 2008/07/18]
7. Pittas F, Ponsonby AL, van der Mei IA, et al. Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis. *Journal of neurology* 2009;256(4):577-85. doi: 10.1007/s00415-009-0120-2
8. D'Hooghe M B, Haentjens P, Nagels G, et al. Alcohol, coffee, fish, smoking and disease progression in multiple sclerosis. *European journal of neurology : the official journal of the European Federation of Neurological Societies* 2012;19(4):616-24. doi: 10.1111/j.1468-1331.2011.03596.x [published Online First: 2011/11/29]
9. Confavreux C, Hutchinson M, Hours MM, et al. Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *The New England journal of medicine* 1998;339(5):285-91. doi: 10.1056/NEJM199807303390501
10. Vukusic S, Hutchinson M, Hours M, et al. Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain : a journal of neurology* 2004;127(Pt 6):1353-60. doi: 10.1093/brain/awh152
11. Hughes SE, Spelman T, Gray OM, et al. Predictors and dynamics of postpartum relapses in women with multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2014;20(6):739-46. doi: 10.1177/1352458513507816 [published Online First: 2013/10/11]
12. Finkelsztejn A, Brooks JB, Paschoal FM, Jr., et al. What can we really tell women with multiple sclerosis regarding pregnancy? A systematic review and meta-analysis of the

literature. *BJOG* 2011;118(7):790-7. doi: 10.1111/j.1471-0528.2011.02931.x
13. van der Mei I, Lucas RM, Taylor BV, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2016;22(4):461-9. doi: 10.1177/1352458515594040 [published Online First: 2015/07/23]
14. Manouchehrinia A, Tench CR, Maxted J, et al. Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain : a journal of neurology* 2013;136(Pt 7):2298-304. doi: 10.1093/brain/awt139 [published Online First: 2013/06/13]
15. Goodin DS. Survey of multiple sclerosis in northern California. Northern California MS Study Group. *Multiple sclerosis (Houndmills, Basingstoke, England)* 1999;5(2):78-88. [published Online First: 1999/05/21]
16. Weiland TJ, Hadgkiss EJ, Jelinek GA, et al. The association of alcohol consumption and smoking with quality of life, disability and disease activity in an international sample of people with multiple sclerosis. *Journal of the neurological sciences* 2014;336(1-2):211-9. doi: 10.1016/j.jns.2013.10.046 [published Online First: 2013/12/03]
17. Paz-Ballesteros WC, Monterrubio-Flores EA, de Jesus Flores-Rivera J, et al. Cigarette Smoking, Alcohol Consumption and Overweight in Multiple Sclerosis: Disability Progression. *Archives of medical research* 2017;48(1):113-20. doi: 10.1016/j.arcmed.2017.03.002 [published Online First: 2017/06/05]
18. Jelinek GA, De Livera AM, Marck CH, et al. Associations of Lifestyle, Medication, and Socio-Demographic Factors with Disability in People with Multiple Sclerosis: An International Cross-Sectional Study. *PloS one* 2016;11(8):e0161701. doi: 10.1371/journal.pone.0161701 [published Online First: 2016/08/26]
19. Diaz-Cruz C, Chua AS, Malik MT, et al. The effect of alcohol and red wine consumption on clinical and MRI outcomes in multiple sclerosis. *Multiple sclerosis and related disorders* 2017;17:47-53. doi: http://dx.doi.org/10.1016/j.msard.2017.06.011
20. Snook EM, Motl RW. Effect of exercise training on walking mobility in multiple sclerosis: a meta-analysis. *Neurorehabilitation and neural repair* 2009;23(2):108-16. doi: 10.1177/1545968308320641 [published Online First: 2008/10/25]
21. Goodin DS, Reder AT, Bermel RA, et al. Relapses in multiple sclerosis: Relationship to disability. *Multiple sclerosis and related disorders* 2016;6:10-20. doi: 10.1016/j.msard.2015.09.002 [published Online First: 2016/04/12]
22. Simpson S, Jr., Blizzard L, Otahal P, et al. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *Journal of neurology, neurosurgery, and psychiatry* 2011;82(10):1132-41. doi: 10.1136/jnnp.2011.240432
23. Comi G, Radaelli M, Soelberg Sorensen P. Evolving concepts in the treatment of relapsing multiple sclerosis. *Lancet* 2016 doi: 10.1016/s0140-6736(16)32388-1 [published Online First: 2016/11/28]
24. Bermel RA, Weinstock-Guttman B, Bourdette D, et al. Intramuscular interferon beta-1a therapy in patients with relapsing-remitting multiple sclerosis: a 15-year follow-up study. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(5):588-96. doi: 10.1177/1352458509360549 [published Online First: 2010/02/20]
25. Bermel RA, You X, Foulds P, et al. Predictors of long-term outcome in multiple sclerosis patients treated with interferon beta. *Annals of neurology* 2013;73(1):95-103. doi: 10.1002/ana.23758
26. Ford C, Goodman AD, Johnson K, et al. Continuous long-term immunomodulatory therapy in relapsing multiple sclerosis: results from the 15-year analysis of the US

prospective open-label study of glatiramer acetate. *Multiple sclerosis (Houndmills, Basingstoke, England)* 2010;16(3):342-50. doi: 10.1177/1352458509358088 [published Online First: 2010/01/29]
27. Goodin DS, Jones J, Li D, et al. Establishing long-term efficacy in chronic disease: use of recursive partitioning and propensity score adjustment to estimate outcome in MS. *PloS one* 2011;6(11):e22444. doi: 10.1371/journal.pone.0022444 [published Online First: 2011/12/06]
28. Goodin DS, Reder AT, Ebers GC, et al. Survival in MS: a randomized cohort study 21 years after the start of the pivotal IFNbeta-1b trial. *Neurology* 2012;78(17):1315-22. doi: 10.1212/WNL.0b013e3182535cf6 [published Online First: 2012/04/13]
29. Goodin DS, Traboulsee A, Knappertz V, et al. Relationship between early clinical characteristics and long term disability outcomes: 16 year cohort study (follow-up) of the pivotal interferon beta-1b trial in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 2012;83(3):282-7. doi: 10.1136/jnnp-2011-301178 [published Online First: 2011/12/24]
30. Scalfari A, Neuhaus A, Degenhardt A, et al. The natural history of multiple sclerosis, a geographically based study 10: relapses and long-term disability. *Brain : a journal of neurology* 2010;133(7):1914-29. doi: 10.1093/brain/awq118